IMPROVING PREVENTION, DETECTION, AND TREATMENT OF INFECTIONS IN NEONATES: SECONDARY ANALYSES OF THE AETIOLOGY OF NEONATAL INFECTION IN SOUTH ASIA (ANISA) STUDY

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Epidemiology in the Gillings School of Global Public Health.

Chapel Hill
2019

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ABSTRACT

Melissa Lynne Arvay: Improving Prevention, Detection, and Treatment of Infections in Neonates: Secondary Analyses of the Aetiology of Neonatal Infection in South Asia (ANISA) Study
(Under the direction of Steven Meshnick)

Neonatal mortality accounts for almost half of deaths in children under age five worldwide. Etiologic agents of neonatal infection are difficult to identify due to non-specific symptoms. We conducted a secondary analysis of data from the Aetiology of Neonatal Infections in South Asia (ANISA) cohort to describe the spectrum of infectious etiologies of acute neonatal illness and assessed association of individual clinical signs with bacterial versus viral infection.*Ureaplasma* infections typically affect pregnant women and very preterm infants. Unexpectedly, *Ureaplasma* was a leading cause of infection among young infants in ANISA. We present and evaluate alternative explanations for this finding.

We applied a partial latent class Bayesian model (pLCM) using a Gibbs sampler to estimate the prevalence of 27 pathogens detectable on a molecular polymerase chain reaction (PCR) platform in sick young infants. We estimated prevalence in different subsets of infants meeting three WHO case definitions We calculated the prevalence for common danger signs among infants meeting the clinical severe case definition. We used observed diagnostic and pLCM results from ANISA to present and evaluate potential alternative explanations of the *Ureaplasma* finding.

Proportion of illness attributable to bacterial infection was 33%, 14%, and 9% among infants in the highest to lowest severity illness WHO case definitions. Among specimens that

tested positive for *Ureaplasma*, 93% speciated to *U. parvum* or *U. urealyticum*. *Ureaplasma* test results did not possess any of the documented criteria that impact the reliability of pLCM model estimates. *Ureaplasma* attribution was twice as high among infants who died (15%) when compared to infants who survived (7%) (P-value <0.0001).

Clinical criteria do not clearly differentiate bacterial from viral etiology among young infants with possible severe infection. Given the fact that *Ureaplasma* is present in term infants, and the fact that we did not identify another viable explanation for the ANISA *Ureaplasma* finding, it is plausible to suggest *Ureaplasma* has been under-detected in previous etiology studies. Further research into *Ureaplasma* as a causative agent of illness in neonateswould inform whether changes to current antibiotic regimens recommended by WHO for treatment of ill neonates are needed.

ACKNOWLEDGEMENTS

I'll never forget the wise words of my first committee chair, Bill Miller, who told me, "This is not about you, it's about the research. Don't take constructive criticism personally, and don't be afraid to ask for help when you need it. It's that help that's going to make the research better and contribute to the body of knowledge". There have been so many people I would like to thank who have shaped this work, and shaped me as an epidemiologist. First, I would like to thank my dissertation chair, Steve Meshnick, for his optimism and his expertise, and most importantly his appreciation for the fact that I was not a "typical" UNC EPID student. I would like to thank the rest of my dissertation committee, especially Daniel Westreich, who not only serves as the methodologist on my committee, but has been my friend for 30 years. He always takes the time to explain complex concepts in a way I will understand, and is happy to lend an ear for non-epi related conversations too. I never would have predicted our paths would intertwined in the way they have, and I am grateful. Thank you to Melissa Bauserman and David Weber for their invaluable advice on the clinical aspects of my research, and thank you to Nong Shang for the countless hours (really years) he has spent developing the ANISA methods, and educating me on them.

A special thanks to my classmates in the UNC Department of Epidemiology, especially Chris Gray, Joann Gruber, Nalyn Siripong, Marissa Seamans, Katie Lesko, and Elizabeth Cromwell for their invaluable friendship and support during and after our graduate education. I look forward to sharing lifelong friendships with each of them.

Thank you to my stellar husband Doug Beard, who has unfailingly supported me, and picked up the slack with parenting our daughter Adelaide on many late nights and weekends. It has been a rollercoaster for the past 4 years, and I could not have done this without you. Thank you to my parents, Joseph and Kathy, who have loved me and supported me in every way possible, in every endeavor, even if they didn't always agree with what I was doing. And my sister Brooke, who is always right by my side at life's important moments.

I would not have these data, or the mechanism to return to school later in life, without the help and support of my CDC mentors, Stephanie Schrag and Bob Pinner. They both constantly challenge me to become a better epidemiologist, and I owe them much of my career accomplishments.

Lastly, I would like to thank all of the study personnel in Bangladesh, India, and Pakistan who worked so tirelessly for years to collect this valuable data. I dedicate this work to the parents who have suffered the loss of a newborn.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFRINEST	Simplified Antibiotic Regimens for Neonatal Sepsis Trials in Africa
ANISA	Aetiology of Neonatal Infections in South Asia
CDC	Centers for Disease Control and Prevention
CHW	Community Health Worker
CI	Credible Intervals (Bayesian)
cOR	Crude Odds Ratios
FPR	False Positive Rate
GBS	Group B S*treptococcus*
IMCI	Integrated Management of Childhood Illness
MCMC	Markov Chain Monte Carlo
NPV	Negative Predictive Value
NP	Nasopharyngeal
OP	Oropharyngeal
PCR	Polymerase Chain Reaction
PERCH	Pneumonia Etiology Research for Child Health
pLCM	Partial Latent Class Analysis Model (Bayesian)
PPV	Positive Predictive Value
pSBI	Possible Serious Bacterial Infection
SATT	Simplified Antibiotic Treatment Trials
TAC	Taqman Low Density Array Cards

TPR	True Positive Rate
US	United States
WHO	World Health Organization

CHAPTER 1: SPECIFIC AIMS

Neonatal mortality accounts for almost 40% of under-five child deaths worldwide. [1-6] More than a third of neonatal deaths occur in Bangladesh, India, and Pakistan; severe infections are among the leading causes. Neonates with suspected infection are difficult to evaluate due to non-specific symptoms and limited diagnostic capabilities. Young infants are often assessed, either by physicians or community health workers using the World Health Organization (WHO) Young Infant Integrated Management of Childhood Illnesses (IMCI) possible serious bacterial infection (pSBI) clinical algorithm. [7-10] The pSBI case definition was designed using limited etiologic data to be sensitive rather than specific to minimize missed opportunities for intervention. However, it results in hospitalization and treatment (a recommended 10 day course of injection or intravenous antibiotics) of some infants without serious infection, and presents implementation challenges in settings where hospital referral is commonly refused.

New evidence that simplified home treatment regimens including reduced antibiotic injections are effective when hospitalization was refused, and supporting limited oral antibiotic treatment of young infants with fast breathing as their only sign, formed the basis of revised guidelines and case definitions in 2015. The guidelines further stratified infants meeting the pSBI case definition into a more severe ("critically ill") and less severe ("clinical severe infection") case definitions. The critically ill infants are now recommended aggressive antibiotic treatment in a hospital setting after receiving pre-referral antibiotic treatment, while the clinical severe cases are recommended simpler antibiotic regimens when referral to a hospital is not possible. Lastly, the infants with late onset illness (=>3 days and < 59 days) with isolated fast

breathing are recommended to receive oral antibiotic treatment with no hospital referral. Much of the evidence used to inform these guidelines was graded as low quality evidence, and a key data gap was comprehensive diagnostic evaluation of infants in the different case strata to allow for a link between infectious etiologies and clinical management recommendations.

One of the key findings from the ANISA study was a high proportion of *Ureaplasma spp.* infections among the infants that met the pSBI case definition. It was the second highest proportion estimated of any pathogen in the study. While *Ureaplasma* spp. is a known relevant infection for neonates, and in particular extremely and very preterm neonates, this finding was unexpected. Here we used the ANISA observed and modeled etiology results to describe *Ureaplasma* as a cause of infection in neonates. We present and evaluate potential alternative explanations of the ANISA *Ureaplasma* finding, and characterized demographics and clinical presentation of infants with infection attributable to *Ureaplasma*.

We used a cohort of more than 5000 possible serious infection cases from South Asia (the Aetiology of Neonatal Infections or ANISA cohort) with diagnostic testing results for bacteria and viruses in blood and respiratory samples to describe the proportion of infants with infectious etiologies of their illness with each of the IMCI danger signs. We will also evaluate the etiologic spectrum represented by the revised case definitions for critically ill children, those with clinical severe infection, and those with fast breathing as their only clinical sign in the late onset period in Bangladesh. Lastly, we will investigate the ANISA *Ureaplasma* finding, and characterize demographics and clinical presentation of infants with infection attributable to *Ureaplasma*.

Specific aim #1a: Describe the distribution of Integrated Management of Childhood Illness Case definitions clinical signs among the infants from the ANISA cohort with infectious

etiologies. We will use the posterior probabilities from the parent study etiology model to calculate the distribution of clinical signs among the infants who had an identifiable infectious etiology, using the Basic Bayesian formula.

Objective: Ascertain if there are differences in the distribution of clinical danger signs among those infants who had a known infectious etiology of their illness.

Hypothesis: The distribution of clinical signs among those with an infectious etiology in the ANISA study will have a different distribution when compared with cases with no identified infectious etiology.

Specific aim #1b: Evaluation of etiologies captured by Integrated Management of Childhood Illness Case definitions for neonatal infections. We will use a Bayesian partial latent class model using a Gibbs sampler to estimate the individual and population level proportions for each pathogen under study in the original ANISA cohort who met the clinically severe case definition, the first case definition under evaluation. Additionally, we will estimate the pathogen proportions among those that meet the critically ill case definition when compared with the clinically severe group, and estimate the pathogen proportions for those infants with fast breathing as their only sign in the late onset period (>7 days) when compared with the clinically severe group, in the Bangladesh site only. We will empirically assess the change by looking for confidence interval overlap.

Objective: Provide evidence that the WHO revisions to the case definitions for possible serious infection in neonates are sound.

Hypotheses: Infants with a clinical severe infection will show similar proportions of infectious etiologies as cases meeting the ANISA case definition. Critically ill infants will have a higher proportion of infectious etiologies when compared with clinical severe cases. Infants

with fast breathing as a single sign in the late onset period (7-59 days) will show similar or lower proportions of infectious etiologies when compared with clinical severe cases in Bangladesh.

Specific aim #2: Understand the demographic characteristics and risk factors associated with *Ureaplasma spp.* infection in neonates in this population of infants in South Asia. We will use the observed diagnostic results data and the partial latent class Bayesian model (pLCM) results from the primary ANISA etiology study to present and evaluate potential alternative explanations for why *Ureaplasma* was the second most prevalent etiology of illness in the ANISA population. We seek to characterize infants with infection attributable to *Ureaplasma* by calculating the prevalence of selected demographics and clinical variables of interest using the standard Bayesian formula, stratified by preterm vs term and early and late onset of disease.

Objective: Describe the infant population with illness attributed to *Ureaplasma spp.*, and present and evaluate potential alternative explanations of the ANISA *Ureaplasma* finding.

Hypothesis: There will be no other viable explanation for the *Ureaplasma* finding, and it is plausible to suggest that *Ureaplasma* has been under detected in previous etiology studies.

CHAPTER 2: BACKGROUND

Most developing countries have witnessed substantial declines in mortality among children <5 years of age [5 11]. In contrast, neonatal mortality rates have remained relatively consistent, with an estimated 3.6 million annual neonatal deaths globally[11-1314,15]. Neonatal mortality accounts for about 40-50% of under-five child deaths. [14-16] Neonatal infections, including sepsis, pneumonia and meningitis account for an estimated 1.4 million neonatal deaths worldwide every year. [6,13] About 60% of the deaths due to infections occur in the first week of life,[1] and therefore, there is only a narrow window of opportunity to intervene. The risk of death in the second month of life is also high, and a vast majority of these deaths occur in the poorest countries of Asia and Africa. [4] Three South Asian countries, Bangladesh, India and Pakistan, account for more than a third of all global neonatal deaths, and a disproportionate portion of deaths in the second month of life. [17,18] The proportion of all child <5 deaths in South Asia increased from 45% to 57% between 1990 and 2015. [17] The majority of births and deaths in these countries occur at home. [19-26]A variety of preventive approaches, including maternal immunization [27], clean childbirth practices, emollient therapy [28,29], promotion of exclusive breastfeeding, and chlorhexidine cord cleansing [30] hold promise in reducing the total number of infection-related young infant deaths. In addition, other investigators have demonstrated the feasibility and effectiveness of identifying young infants with serious infection using clinical algorithms, and safely treating them at the community level. [8,10,13,31-33]

Several antibiotic regimens are currently used to treat young infant infections and other regimens are under evaluation. [34 33] Most treatments, however, are presumptive and the diagnosis

is based on clinical algorithms which are not specific for invasive bacterial infection. [9,34,35] Although treatment regimens currently utilized can reduce neonatal mortality by up to 50% [13,31,33,36], appropriate formulation of antibiotic treatment regimens is hampered by lack of data on etiology and antibiotic susceptibility patterns. A single regimen may not be optimal for management of newborn infections in different geographic regions since the etiology of infections and antimicrobial susceptibility patterns of major isolates may vary across settings. Moreover, antibiotic regimens that are not targeted for the leading young infant pathogens may contribute to treatment failures and emergence of multidrug-resistant bacteria in the community.

Etiology of young infant infections in developing countries has been described in only a few studies, where 5-10% of cases of suspected serious infections were positive for any bacterial etiology by blood culture. [2,24,37-41] Etiology of more than 90% of suspected infection cases remains unknown. The ANISA etiology study found very similar results, with 15.8% and 12.2% of cases attributed to bacteria and viruses, respectively. [42] A proportion of these cases may be caused by bacteria or viruses that are currently unrecognized as causes of young infant infection. Additionally, most of these studies did not capture infants during the first 3 days of life, a period of high mortality. Notably, these studies did not collect specimens from healthy infants to act as a control group. Finally, most studies were conducted in hospital settings; because of low care-seeking and inclusion of nosocomially-acquired infections, the pathogen distributions in these studies are unlikely to be representative of infections in the community.[25] Thus, despite the large burden of neonatal deaths in the community attributable to infections, there are almost no data on the etiology of community-acquired young infant infections, [2,3]or geographic variation in etiology. [24,43]

Clinical algorithms are useful to identify sick newborns with severe infection, particularly in settings where laboratories are not readily available. A 1999 community-based sepsis study conducted in rural India used the simultaneous presence of any two of seven signs: poor sucking, weak or no cry, limp limbs, vomiting or abdominal distension, cold to touch, retracting, umbilical infection.[31] These indicators predicted presumed sepsis death in newborns less than one month of age with sensitivity of 100% and specificity of 92%. The criteria identified 10.6% of the neonates in the community with suspected cases of sepsis. [44] The Young Infants Clinical Signs Study Group in 2008 evaluated infants 0-6 days old and 7-59 days in outpatient health facilities and used the presence of any one of seven signs: history of difficulty feeding, history of convulsions, movement only when stimulated, respiratory rate of 60 breaths per minute or more, movement only when stimulate, severe chest in-drawing, temperature of 37.5°C or more or below 35.5°C. This study used these signs to predict an expert pediatrician panel's clinical judgment of severe illness, requiring referral to a hospital for admission. The prediction model had a sensitivity of 85% and specificity of 75%. [10]

Recently, studies have validated community health workers' (CHW) assessments of severe young infant disease using clinical algorithms comprised of similar signs as those that will were used in the ANISA study, compared to physician assessment as the gold standard. A 2009 study documented CHW's classification of very severe disease using a simple clinical algorithm with sensitivity of 73%, specificity of 98%, positive predictive value of 57% and negative predictive value of 99%.[8] Similarly, another study the same year found CHW's classification of very severe disease using a similar clinical algorithm to have a sensitivity of 91%, specificity of 95%, PPV of 51% and NPV of 99%. [7]

Evidence for revision of guidelines for treatment: trials of simplified antibiotic regimens for the treatment of infections in neonates

In recent years, there has been a set of trials using standardized protocols to test the use of simplified antibiotic regimens that can be provided in low resource settings where referral to an inpatient facility or hospital may not be possible. Three of the trials (SAT-Bangladesh, SAT-Pakistan and AFRINEST-severe infections) studied whether young infants with clinical signs indicative of possible severe infection can be treated with a combination of oral amoxicillin plus gentamicin, and whether injections can be stopped after the first 2 days with a transition to oral amoxicillin alone for 5 days. This could effectively reduce the number of injections given and making treatment regimens simpler to follow. These equivalency trials examined whether adherence to regimens will differ between simpler antibiotic regimens when compared with the standard regimen of procaine penicillin and gentamicin injections daily for 7 days, using a predefined margin of +/- 5%.

The results of the SAT-Pakistan trial per protocol analysis of 2780 infants showed that there was no significant reported risk difference in treatment failure within 7 days of enrollment between infants in the three treatment arms--the reference treatment (intramuscular procaine benzylpenicillin and gentamicin) when compared with the simplified treatment regimens of oral amoxicillin and gentamicin (risk difference: -1.9% (-5.1%, 1.3%) and procaine benzylpenicillin, gentamicin, and amoxicillin (risk difference: 1.1% (-2.3%, 4.5%).[46] Similarly, the results of the SAT-Bangladesh trial also suggest that the same two simplified treatments were equivalent to the reference treatment of intramuscular procaine benzylpenicillin and gentamicin, with risk differences of -1.5% (-4.3%, 1.3%) and -1.7% (-4.5%, 1.1%), respectively. [47]

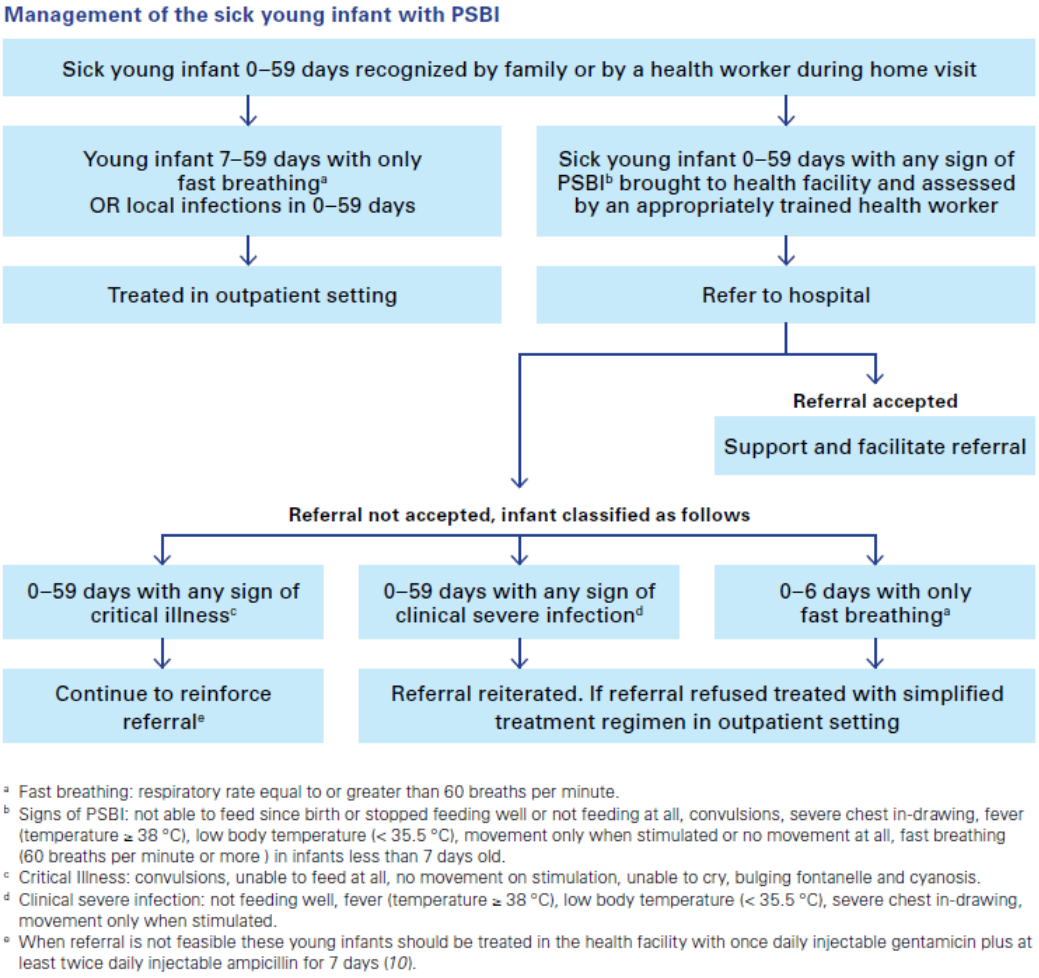
The AFRINEST multisite equivalence trial was conducted in the Democratic Republic of Congo, Kenya, and Nigeria. [45] Neonates were visited by CHWs and assessed for any signs or symptoms of illness. The infants were stratified by age of onset, and were randomized to one of four treatments: intramuscular procaine benzylpenicillin-gentamicin for 7 days (reference); intramuscular gentamicin and oral amoxicillin for 7 days (risk difference: -1.9% (-4.4%, 0.1%); intramuscular procaine benzylpenicillin-gentamicin for 2 days followed by oral amoxicillin for 5 days (risk difference: -0.6% (-3.1%, 2.0%); and intramuscular gentamicin for 2 days and oral amoxicillin for 7 days (risk difference: -2.7% (-5.1%, 0.3%) All three simplified regimens were found to be as effective as the reference treatment of intramuscular procaine benzylpenicillin-gentamicin for 7 days when administered on an outpatient basis.[45]

Fast breathing as the only sign of illness among infants 0-59 days of age

The AFRINEST trial also studied equivalence of simplified antibiotic regimens in infants aged 0-59 days of age with fast breathing as their only sign, where their parents did not accept referral to a hospital[48]. In this equivalency trial, 2196 infants were randomized in a per protocol analysis to either 1. Oral amoxicillin for 7 days or 2. A combination of intramuscular gentamicin plus procaine penicillin for 7 days. The equivalency was defined as having a risk difference between +/- of 5%. This study compared four different outcomes between the intervention group and the control group. The first was treatment failure in the first week after enrollment in the study. There was no significant difference between the 2 groups in this outcome, with a risk difference of -2.6% (-6.0%, 0.8%), although the lower limit of the confidence interval around the risk difference was lower than the equivalency margin. [49] The second was incidence of death by the 15th day after enrollment. Again, the results showed equivalency between the two groups with an estimated pooled risk difference of -0.02% (-

0.5%%, 0.5%). The third was relapse of illness as defined by fast breathing as the only sign during the second week after enrollment. As with the other outcomes, the two groups showed equivalency with a pooled risk difference of -0.02% (-1.2%, 1.6%). Lastly, the fourth was 100% adherence to the scheduled doses of antibiotics on all 7 days or until the treatment failed. Adherence (defined as 100% of doses on all 7 days) was found to be better among the simplified regimen intervention group (98.5%) when compared with the reference regimen (90.7%). [49]

Figure 1. Revised algorithm for the management of sick young infants with possible serious infection, Guidelines for managing possible serious bacterial infection in young infants when referral is not feasible, World Health Organization, 2015. [50]



[a] Fast breathing: respiratory rate equal to or greater than 60 breaths per minute.
[b] Signs of PSBI: not able to feed since birth or stopped feeding well or not feeding at all, convulsions, severe chest in-drawing, fever (temperature ≥ 38 °C), low body temperature (< 35.5 °C), movement only when stimulated or no movement at all, fast breathing (60 breaths per minute or more) in infants less than 7 days old.
[c] Critical Illness: convulsions, unable to feed at all, no movement on stimulation, unable to cry, bulging fontanelle and cyanosis.
[d] Clinical severe infection: not feeding well, fever (temperature ≥ 38 °C), low body temperature (< 35.5 °C), severe chest in-drawing, movement only when stimulated.
[e] When referral is not feasible these young infants should be treated in the health facility with once daily injectable gentamicin plus at least twice daily injectable ampicillin for 7 days (*10*).

These data provided some evidence for the recent revision of the guidelines for managing possible serious bacterial infection in young infants when referral is not possible. The new guidelines further stratified infants meeting the possible serious bacterial infection (pSBI) case definition into a more severe ("critically ill") and less severe ("clinical severe") case definitions. The critically ill infants are now recommended aggressive antibiotic treatment in a hospital setting after receiving pre-referral antibiotic treatment, while the clinical severe cases are recommended simpler antibiotic regimens when referral to a hospital is not possible. Lastly, the infants > 7 days with fast breathing as their only sign are recommended to receive oral antibiotic treatment with no hospital referral (Figure 1).

Much of the evidence used to inform these guidelines was graded as low quality evidence, and a key data gap was comprehensive diagnostic evaluation of infants in the different case strata to allow for a link between infectious etiologies and clinical management recommendations.[50] The important risk factors and modes of transmission for young infant infections also remain poorly understood. [51] Most studies have been conducted in developed countries, and has focused on early-onset neonatal sepsis and, even more specifically, group B S*treptococcus* (GBS).

Evaluations of all-cause neonatal sepsis have commonly identified preterm delivery, low gestational age, intrapartum fever, chorioamnionitis, and prolonged ruptures of membrane as risk factors for early-onset sepsis. [52-54] The risk of early-onset neonatal sepsis also increases with number of vaginal exams during labor [53 54] An evaluation limited to early-onset invasive *Escherichia coli* infections identified low gestational age, prolonged rupture of membrane and intrapartum fever as the primary factors associated with increased risk. Risk factors for early-onset GBS infections include maternal GBS colonization, intrapartum fever, preterm delivery,

prolonged rupture of membrane, having had a previous infant with GBS disease, and black race. [55-57] Described factors associated with late-onset GBS sepsis are similar to early-onset sepsis, with preterm delivery as a stronger risk factor (50% of late onset cases compared to 25-30% of early onset cases). [58] However, environmental factors such as nosocomial transmission, person-to-person transmission, and transmission via breast milk, may play an important role.

One notable result of the ANISA etiology study was the high proportion attributable to *Ureaplasma* spp. infection. [42] *Ureaplasma spp.* are bacteria in the class *Mollicutes*, and are some of the smallest self-replicating microorganisms, with regard to cellular dimensions and genomic size. [59] Their small genomes result in part to the complex requirement for growth media in vitro, making them difficult to culture. [59] *Ureaplasma* infections are typically associated with adult disease, usually manifesting in upper urinary tract infections, or conditions complicating pregnancy, such as preterm labor, premature rupture of membranes, spontaneous term labor, and chorioamnionitis. [59-65] *Ureaplasma* has been identified in cerebral spinal fluid (CSF) of preterm infants presenting with hydrocephalus, sepsis, and meningitis. [65] In preterm infants, an association between *Ureaplasma urealyticum* and short and long term pulmonary morbidity, as well as mild cerebral impairment has also been described.[66] *Ureaplasma*-associated neonatal infections primarily occur among preterm infants, and is not commonly reported as a pathogen causing neonatal illness in term infants, even in high income countries where data are more readily available.[59,67]

Ureaplasma spp. were identified in higher numbers in infants with suspected infection compared to healthy infants. Crude odds ratios (cORs) varied by specimen type and study site, ranging from 1.5 to 2.7. When stratified by age at onset of disease, cORs were higher for infants

with early onset disease. cORs were 3.7 and 1.7 (p-value=<0.0001) for respiratory specimens and 1.4 and 1.3 for blood specimens among infants who died vs survived, respectively.

The overall proportion of infants meeting the ANISA possible serious bacterial infection (pSBI) case definition with infections attributable to *Ureaplasma* spp. was 2.82% (1.93, 3.77%), the second highest proportion after respiratory synctitial virus (RSV), in a study where most of the pathogens were each responsible for <1% of infection. There was not a significant difference in attribution between infants with early and late onset disease, but the proportion of attributable illness to *Ureaplasma* was higher among infants who died (8.3% (4.1, 12.3)) vs those who survived (2.7% (1.8,3.7)).

While *Ureaplasma* spp. is recognized in the literature as a relevant infection for preterm neonates, the ANISA finding was unexpected. While the ANISA cohort had 28% of sick infants with a gestational age of less than 37 weeks, only 2.9% of them were either extremely or very preterm (< 32 weeks of age). Furthermore, the *Ureaplasma* finding was not limited to preterm infants. The cORs for *Ureaplasma* was higher in term infants than it was for preterm infants, at 1.8 vs. 1.6 in respiratory specimens, and 1.5 and 1.3 for blood specimens, respectively.[68]

A study using very similar methodology to ANISA conducted in infants in the first 3 days of life in Soweto, South Africa also found *Ureaplasma* to be the leading etiology proportion (5.4%) among neonates under study. [69]

CHAPTER 3: METHODS

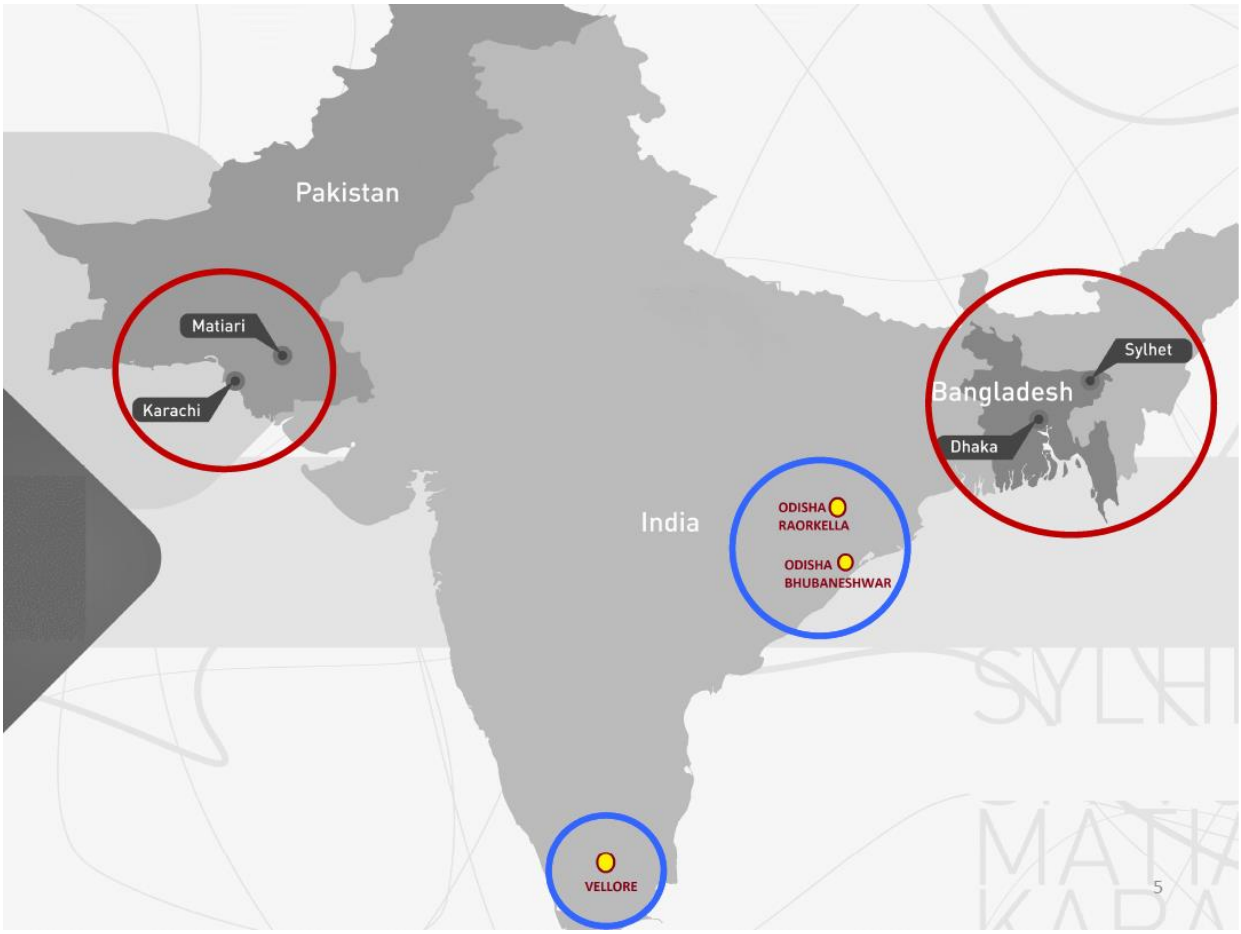
Parent Study Design and Population

The Aetiology of Neonatal Infections in South Asia (ANISA) project, a multi-center, population based longitudinal study whose objective was to determine the population-based incidence, etiology and antibiotic resistance profiles of community-acquired young infant infections in Bangladesh, India and Pakistan, was conducted from 2010-2014 (Figure 2). This study followed women of child bearing age in defined catchment areas for pregnancy and birth outcomes. Upon birth, each child was assessed for illness at 10 scheduled visits using the IMCI case definition for possible serious bacterial infection during the first 60 day of life. Infants less than 60 days with at least one of the signs (history of convulsions, respiratory rate > 60/min, severe chest in drawing, axillary temp either >99.5 degrees F or <95.9 degrees F, little or no movement, and poor feeding) resulted in hospital referral and a regimen of either parenteral or oral antibiotics. Infants with fast breathing as their only sign were not sampled routinely except in the Sylhet site. In the analytic stage, an algorithm was used to ensure that an infant was considered a new case if the study physician confirmed that 1) the child met one of the above signs, 2) had not been hospitalized in the preceding 7 days (excepting post-natal hospital stays of facility-born infants), and 3) had not been previously enrolled as a case in prior 7 days. In the etiology study analysis, all cases who only had elevated respiratory rate were excluded.

There were three major assumptions made when designing the parent study. CHWs can assess the signs with sufficient precision to identify sick neonates most likely to have a laboratory confirmed invasive bacterial infection. The ANISA case definition (IMCI criteria for

very severe disease) is sensitive enough to capture all cases of clinically important bacteremia. The laboratory methods are sensitive enough to detect a vast majority of bacteremias requiring antimicrobial therapy.

Figure 2. Map of Aetiology of Neonatal Infections in South Asia (ANISA) Study sites, 2011-14



Source: Dr. Samir Saha, Child Health Research Foundation

A panel of neonatologists with expertise in neonatal infections was convened, and the Delphi method was used to identify a priority list of pathogens for testing of nasopharyngeal (NP) and oropharyngeal (OP) swabs and blood specimens from infants enrolled in the ANISA study[70].

Respiratory (nasopharyngeal and oropharyngeal swabs) and blood were taken from cases to test for presence of pathogens. A subset of healthy infants were visited and enrolled based on

age and date of children who met the case definition. The same specimens were collected from healthy infants in order to provide a comparison group for baseline carriage of bacteria and viruses in the same population. All blood specimens were tested using automated blood culture methods (for cases only) to detect bacteria, and by polymerase chain reaction (PCR) by TaqMan Array card (TAC) panel testing for 27 different bacteria and viruses, and respiratory specimens were tested by PCR TAC panel alone (Appendix 3). [71] Infectious disease specialists reviewed and classified each isolate as a pathogen or contaminant, based on clinical information on the patient, and other laboratory and antibiotic susceptibility data. The study surveillance and laboratory methods have previously been described.[72-78]

Etiology was measured by estimating the probability that each organism class was attributed to a given case of illness. These estimations were based upon the observed blood culture and molecular testing results, using a Bayesian partial latent class model method. [42,79] These organism classes varied by study site, age of onset of illness, data of enrollment, and outcome (died with a specimen available within 7 days of death). Pathogen lists created by a stepwise procedure that was informed by the number of across- class combinations of test results was used, and varied by study site. False positive rates were estimated, and were allowed to vary across the aforementioned covariate levels, but the estimated true positive rates were held constant. Posterior means were reported as the organism class proportions, with corresponding 95% credible intervals (Appendix 4).

Aim 1 Methods

Case definitions

In accordance with WHO case definitions, we defined an infant with "clinical severe infection" as one who presented with at least one of the following signs: severe chest in-drawing, axillary temp either =>38°C or <35.5°C, movement only when stimulated, and failure to feed

well (confirmed on observation by study personnel).[11] We defined a "critically ill" infant as one who presented with at least one of the following signs: lack of consciousness (no movement at all), inability to feed, inability to cry, physician-observed convulsions, apnea, cyanosis, bulging fontanelle, major congenital malformations inhibiting oral antibiotic intake, and persistent vomiting (defined as vomiting following three attempts to feed within 30 minutes). We defined an infant with late-onset isolated fast breathing as one aged 3-59 days with elevated respiratory rate (i.e. >=60/minute) as their only sign. We do not present the results for the early onset (< 3 days of age) infants in this analysis. Each of these case definitions are mutually exclusive. All clinical signs used in these definitions were based on physician's assessment.

Data analysis

A partial latent class model was used to estimate pathogen population proportions for infants with clinical severe infection, and compare these proportions to those for infants who were critically ill and those with isolated late-onset fast breathing. Statistical differences between proportions are defined as overlapping Bayesian 95% credible intervals. The comparison group for the critically ill infants consisted of all infants with clinical severe infection, while the reference group for the infants with late-onset isolated fast breathing was restricted to infants 3-59 days old with clinical severe infection. Our analysis of infants with isolated fast breathing was limited to the Bangladesh site because this was the only site where respiratory and blood specimens were routinely collected from such infants. Our hypothesis was that critically ill infants would have a higher proportion of bacterial etiologies when compared to those with clinical severe infection, and that infants with isolated fast breathing in the late onset period (3–59 days) would have a lower proportions of bacterial etiologies when compared to those in the same age group with clinical severe infection.

Etiologic attribution

ANISA statistical methodology was an extension of the basic partially-Latent Class Model (pLCM) developed by Wu et al for the Pneumonia Etiology Research for Child Health (PERCH) to estimate the proportion of pneumonia infections attributed to one of six specific pathogens.[80] We applied the same partial latent class Bayesian model as used in the primary study, which used a Gibbs sampler to estimate individual and population-level probabilities for each of 27 pathogens detectable on the molecular PCR TAC platform, and an "other/none" category, which included cases not attributed to any etiology.[68,79] The Gibbs sampler is a Markov Chain Monte Carlo (MCMC) algorithm for obtaining a sequence of observations which approximate a specified multivariate probability distribution. Each sequence was used to estimate the latent variables of etiology proportion and test characteristics, i.e. the true positive rate and false positive rate for every test and pathogen. The detections in specimens obtained from healthy infants were used to set priors for the false positive rates in the model. We will use the same stepwise method for selection pathogen lists for incorporation to the model for every site. A non-parametric Bayesian model will be used to adjust these estimates for covariates of age and time of enrollment. Another class was indirectly calculated, i.e. not estimated by the model, for all other bacteria that were detectable by blood culture but did not have a corresponding molecular test. The mean, 2.5th and 97.5th percentiles for the Bayesian credible intervals are reported.

We will hold the six main assumptions made in the primary etiology analysis, which are the following:

- There is only one etiologic pathogen in an episode of illness among those who met the case definition.

- True positive rates will be held constant across site and other covariates (age and date of enrollment) because infection assumes presence of the pathogen in the clinical specimens collected from cases.
- False positive rates will vary across site, age, and date of enrollment.
- Enrolled healthy controls are not ill, and hence any detection of pathogens in clinical specimens is considered carriage.
- We assume conditional independence, in that the carriage of one pathogen will not affect carriage of another pathogen, if neither of them are the cause of the illness.
- We will set lower limits for TAC test true positive rates: 40% for respiratory molecular tests and 20% for Blood molecular tests. We did not set a lower limit for blood culture true positive rates. Blood culture results are held as a gold standard, with the assumption that the true positive rate is 100% and the false positive rate is 0%.

Model specifications

The probability of observing $\{y_{ik}\}$ is expressed by a linear mixture model with $\{\pi_k\}$ as the mixing coefficients. By applying the regular conditional independence assumption for linear mixture class models with unknown latent classes, and a further assumption that the probability of test T_k to produce positive test result depends only on whether pathogen k is the true etiology of the tested case, Wu et al. developed a partially-Latent Class Model (pLCM) [80]:

$$f(y_{ik}, k = 1, \dots, K; i = 1, \dots, N) = \prod_{i=1}^{n}\left(\sum_{k=1}^{K} \pi_k \theta_k^{y_{ik}} (1-\theta_k)^{1-y_{ik}} \prod_{j \neq k} \delta_j^{y_{ij}} \left(1-\delta_j\right)^{1-y_{ij}} + \pi_{K+1} \prod_{j} \delta_j^{y_{ij}} \left(1-\delta_j\right)^{1-y_{ij}}\right)$$

Here parameters $\theta_k = f(y_{ik} = 1 \mid true\ etiology = k)$ and $\delta_k = f(y_{ik} = 1 \mid true\ etiology \neq k)$ are called the True Positive Rate (TPR) and False Positive Rate (FPR) respectively for test $T_k, k = 1, \dots K$. [79,81]

Rationale for using partial latent class analysis model

Determining etiology of neonatal infections, particularly in low resource settings, is complex. Previously, other studies in the literature have conducted descriptive analyses of blood culture results only in an effort to characterize the etiology of neonatal infections.[2] In more recent years, studies such as the Global Enteric Multicenter Study used an attributable etiologic fraction approach. Both of these approaches have a number of limitations. The methods makes implicit assumptions about perfect sensitivity of all laboratory tests, there is no way to combine results for the same pathogen across multiple specimens, there is no way to get an etiologic attribution at the individual level, and there is not a straightforward way to adjust for covariates. [82] In the case of the ANISA study, we had varying number of tests (between 1 and 3) for each of the 27 pathogens selected for the molecular panels, and two different types of specimens (blood and NP/OP swabs). In an ideal world, we would identify infants with serious infections using a case definition with high sensitivity and specificity, we would obtain ideal samples and test them using laboratory tests with high sensitivity and specificity. In reality, the laboratory tests used have imperfect test characteristics. While blood culture, which is a historical "gold standard" and is typically assumed to have 100% sensitivity, it has unknown specificity -- we have no blood culture results from controls to inform specificity. The molecular platform used has been analytically but not clinically validated, [83] so we do not know the test characteristics of these tests. This method was chosen for its ability to incorporate multiple tests per pathogen for a given case into the estimation of pathogen probabilities at an individual and population level, and allowed us to quantify the uncertainty around diagnostic tests. [42,79,84]This decision was informed by another multicenter study estimating the etiology of pneumonia in children under 5 years of age. They developed a partially latent class model which was adapted for the ANISA etiology study. [80,81,84,85] The ANISA primary etiology study has made a number of innovations to this

method, including building in a non-parametric conditional density estimation to control for confounding, and establishing a method for pathogen selection into the model based on the number of novel combinations identified between detections and covariates.

Infants with at least one clinical specimen available were included in the analysis. For the analysis including infants with isolated fast breathing, we used the same true positive rates as estimated by the ANISA etiology model (we set these values as constants, and therefore they were not estimated by the model). The Bayesian partial Latent Class Analysis will be conducted using R! and R! Studio and the clinical signs analysis will be conducted using SAS software version 9.3®. [86,87]

Distribution of clinical signs among cases with infectious etiology

To examine the distribution of clinical signs among infants who met the clinical severe infection case definition and had an infectious etiology, we used the clinical severe infection model output, which generates an estimated probability for every pathogen. We calculated the posterior probability for every sign in the IMCI case definition using the standard Bayesian formula: P(clinical sign|infectious etiology)=P(clinical sign x infectious etiology)/P(clinical sign).[88] We then used the posterior probabilities from every iteration to obtain a distribution for each sign, and reported the median as the point estimate and 2.5^{th} and 97.5^{th} percentiles for the Bayesian credible intervals. We stratified these probabilities by early and late onset of disease. Within these categories, we further stratified by attribution to a bacterial versus viral etiology.

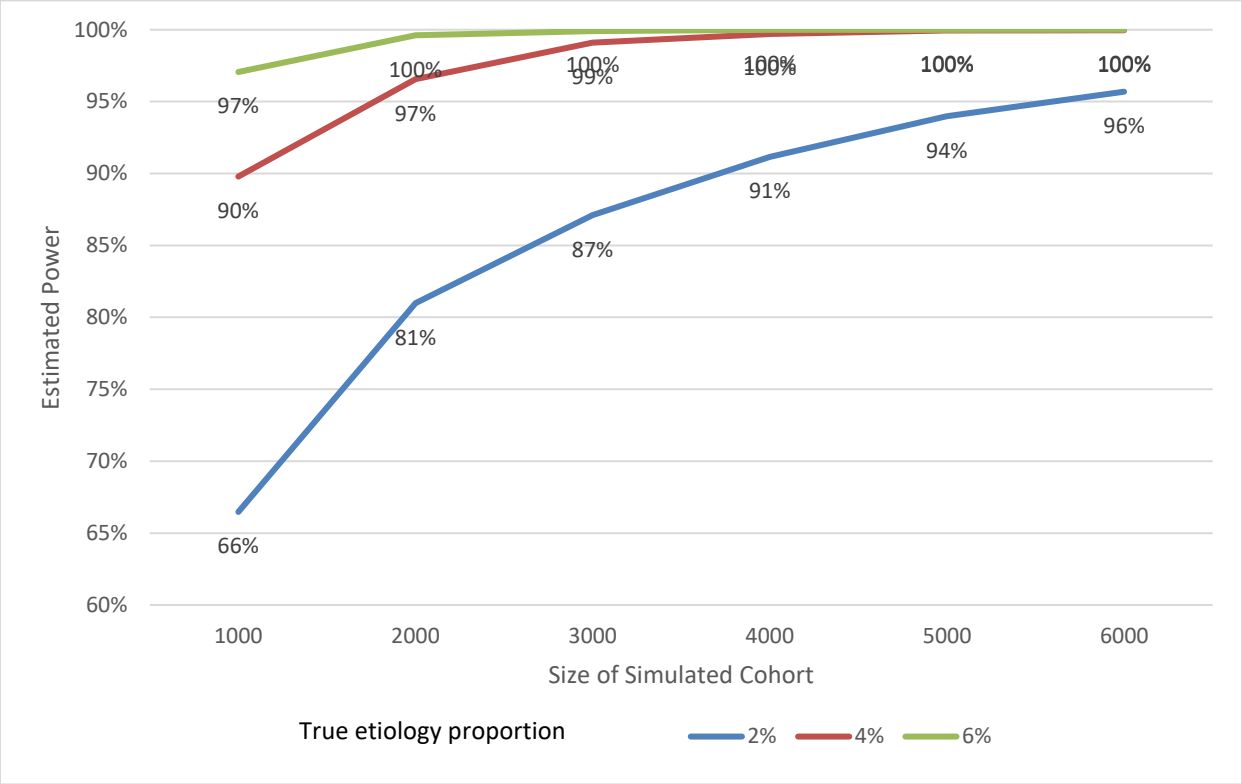
Study power

We are using a cohort from the parent ANISA study, in which 5,253 cases and 1,865 healthy controls were enrolled; hence, our sample size is fixed. There are no known methods that allow to estimate power for a Bayesian partial latent class model. In order to estimate power for this analysis, we estimated what our power would be if we used the population attributable

fraction method.[89] We will use the estimated power as a proxy for power using the pLCM method, with the knowledge that EF method does not taking into account confounding, which decreases power. We ran a simulation of different sized cohorts between 1000-6000 enrolled cases, varying our assumptions on the true etiology rate for one hypothetical pathogen between 2 and 6%. We assumed the true etiology proportion would be +/- 2% of the estimated etiology proportion. We held the true positive rate constant at 80% and the false positive rate constant at 10%. Our etiology fraction formula calculated under these simulated conditions is the following: $\hat{p} = (p_{obs} - FPR) / (TPR - FPR)$.[90]

Our cohort is slightly less than 5000 subjects: the estimated power for a true etiology rate of 2% given a cohort of 5000 is 94% (Figure 3). Note that the higher the true etiology rate, and the larger the cohort, power increases. Even at the lower true etiology proportions and smaller cohort sizes, the smallest estimated power is 64%.

Figure 3. Estimated power curves using the etiologic fraction method under different underlying assumptions about sample size, true etiology proportion, true positive rate, and false positive rate for one hypothetical pathogen



Study limitations

This analysis has a number of limitations. We are limited to the universe of those infants who met the case definition. Ideally, we would run a predictive model to measure which clinical signs best predict the outcome of each infectious etiology; however, we cannot use this method because the outcome itself is defined by the clinical signs. Similar to the main ANISA etiology study, we will set no bounds on the true positive rate for the blood culture sensitivity. That coupled with a much smaller sample size, we believe that the estimated proportion for certain pathogens, such as Salmonella, with a low true positive rate but a good number of detections, may be inflated. There are other criteria included in the critically ill case definition that were not recorded by ANISA study and therefore were not evaluated. These criteria are active bleeding

requiring transfusion, surgical conditions needing hospital referral, and fast breathing day 0-6 (this criterion was evaluated separately in Sylhet only).

Missing data

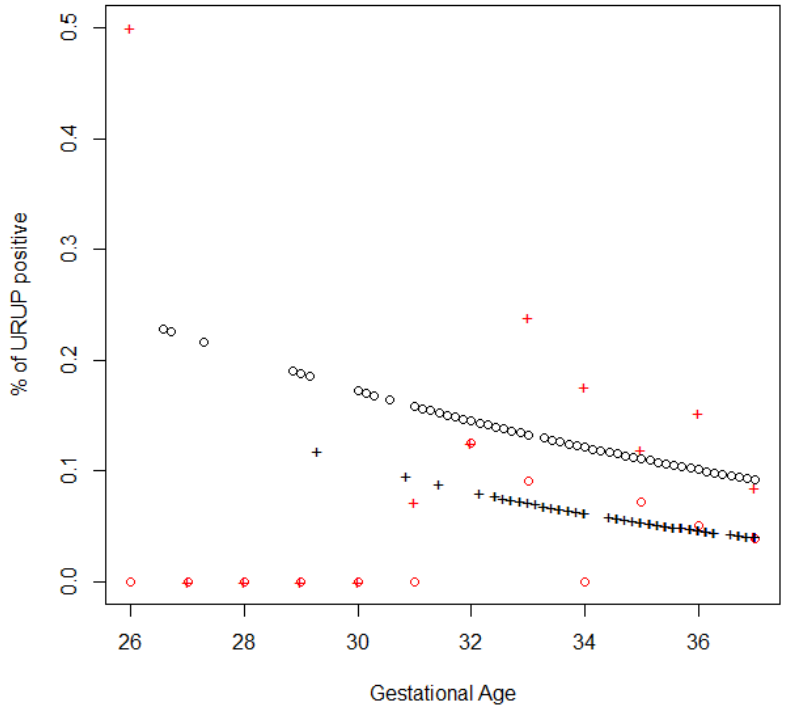
There was a low proportion of cases with missing data (<5%), and we assumed that these data were missing at random. Although considerable effort was made to reach infants on day 0 of their lives, there were many severely ill infants that went on to die who were missed on the first hours of life. Hence, the parent study was only able to collect clinical specimens on 9% of all deaths.

Aim 2 Methods

Preliminary data

We were originally concerned there could be a difference in colonization with *Ureaplasma spp*. based on whether an infant is delivered term or preterm, i.e. if the observed association between presence of *Ureaplasma* bacteria and meeting the case definition might have been driven by over representation of preterm infants among the cases versus controls. We modeled *Ureaplasma* detections as a function of study site, age, and gestational age, with inclusion of an interaction term between gestational age and whether an infant was a case or healthy infant. The data from the linear regression model showed that the detections uniformly decrease as gestational age increases in both groups, and the interaction term was not significant. Therefore, the *Ureaplasma* proportion found in the cases is likely not driven by preterm infants (Figure 4).

Figure 4. Distribution of gestational age among both cases and controls with detections of *Ureaplasma spp.* in their blood and respiratory specimens, Aetiology of Neonatal Infections in South Asia (ANISA) Study, 2011-14



+ healthy infants ° cases

The assay used to detect *Ureaplasma spp.* was analytically validated for serotypes 1, 3, 6, and 14 for *U. parvum* and serotypes 2, 4-5, and 7-13 for *U. urealyticum*. [71] Specificity of each set of primers was evaluated using the Basic Local Alignment Search Tool (BLAST) National Center for Biotechnology Information (NCBI) database. An internal no-template control and a positive control were incorporated into the TAC design, to detect card or individual assay or extraction failures. In the event of a card failure, the card was re-run until the controls indicated valid results. Extraction failures resulted in re-extraction and testing for that specimen. All specimens in which *Ureaplasma spp.* were detected went on to speciation to *U. parvum* or *U. urealyticum* using real-time PCR. [68]

Etiologic attribution methods

We used the etiology results from the ANISA Bayesian model (pLCM) using a Gibbs sampler to estimate individual and population-level probabilities for each pathogen on the molecular PCR TAC platform, and an "other/none" category, which included cases not attributed to any etiology. [68,79-81,84]

The ANISA etiology attribution model was evaluated by simulation studies. [79] These studies identified scenarios, or "danger zones" in which the model does not perform well, e.g. the model will not converge, or estimates are more vulnerable to bias and inaccuracy. For the ANISA pLCM model, these scenarios included: when pathogens have only one laboratory test result available (i.e. only one molecular test); if the true pathogen population proportion is very low (<0.5%); if the crude odds ratio is less than 1; if the false positive rate is high (greater than 75%), or if the true positive rate and false positive rate for that pathogen are close together (less than 25% difference between the two). [79]

Key terms and definitions

A high grade fever of a pregnant woman was defined as a self-reported temperature >38.0`C (>100.4`F). Any complications during pregnancy was defined as having one of the following: high grade fever, foul smelling discharge, swelling of face or feet, excessive bleeding, or convulsions. A skilled provider or skilled birth attendant was defined as a licensed, qualified doctor, nurse, midwife, or paramedic. Maternal poor nutritional status was defined as mid upper arm circumference of less than 21.5 centimeters. Received full antenatal package was defined as: 1. receipt of at least 2 antenatal visits from a community health worker; and 2. Receipt of 2 tetanus toxoid injections during current pregnancy, or 1 tetanus toxoid injection and at least 4 shots pre-pregnancy, or at least 5 tetanus toxoid injections pre-pregnancy; and 3. Receipt of at least one iron tablet or dose or iron syrup during current pregnancy. Delivery at a health facility

was defined as being born at a public or private hospital, a maternity center, or clinic. Any other complication during labor/delivery was defined as excessive bleeding, convulsions, retained placenta, abnormal presentation, prolonged labor, or premature water breaking.

Proper tying and cutting of umbilical cord was defined as use of an acceptable device for both cutting and tying the umbilical cord. Acceptable devices for cutting cords in the home must have been boiled and be either thread, clip kit, knife, blade, or tongs. Acceptable devices for cutting cords in a hospital or health facility must have been nurse/doctor's scissor or clip kit. Acceptable devices for tying cord in the home must have been boiled and be either a clip, clip kit, thin rope brought by doctor, blade, or rubber band. Acceptable devices for tying a cord in a hospital or health facility were a clip or clip kit. Proper application of antibiotic or antiseptic to the umbilical cord differed by setting. In the home, antibiotic or antiseptic must have beenapplied to the stump. In the hospital, antibiotic or antiseptic can be applied, or nothing can be applied. Delayed breath or crying was defined as a delay of either breathing or crying greater than 1 minute after delivery.

A case was defined as a registered infant between 0 and 59 days of age that presented with one of the following signs, as confirmed by a physician: respiratory rate >=60/minute (fast breathing), severe chest in-drawing, axillary temperature either =>38°C (fever) or <35.5°C (hypothermia), movement only when stimulated or no movement at all, convulsions, or not feeding well confirmed on observation by study personnel. A healthy infant was an infant who did not meet the case definition at the time of clinical specimen collection. Early onset disease was defined as illness occurring less than 3 days of age; late onset disease was defined as illness occurring between 3 and 59 days of age. Preterm was defined as an infant with gestational age of less than 37 weeks. Low birth weight is defined as an infant weighing <2500 g at birth.

A positive detection in a case was defined by a positive result on a TAC card from either respiratory or blood specimens. An average individual probability was the likelihood estimated by the ANISA pLCM model that a given organism was the causative agent of illness for a given case. The *Ureaplasma* attributable population is the aggregated proportion of individual probabilities across site and weighted by number of cases enrolled per month in that site. *Ureaplasma* attributable cases were defined as individual infants who met the ANISA case definition, with a probabilistic assignment of *Ureaplasma* as the causative agent of illness, based on the estimated pLCM model results. The true positive rate (TPR) is defined as the probability of having a positive test if the case is caused the pathogen. The false positive rate (FPR) for a pathogen is defined as the probability of having a positive test result for a subject that does not have the pathogen as the etiologic cause of illness.

Concordance between respiratory and blood tests was calculated using the number of infants with detections in both blood and respiratory specimens, divided by the total number of detections in either specimen type.

To examine whether *Ureaplasma* could be construed as a commensal organism co-occurring with other organisms that were the true cause of infection, we compared the observed distribution of pathogen detections between healthy infants and infants with *Ureaplasma* illness. In order to obtain a population of infants with *Ureaplasma* attributable illness, we randomly sampled the *Ureaplasma* individual probabilities generated from the ANISA pLCM model. Random sampling of this output probabilistically assigned a binary value (yes, no) for *Ureaplasma* as the causative agent of illness, [28,29] and we sampled the population 50 times to quantify variability in the estimates. We calculated a mean value with 95% credible intervals for the number of detections across all 50 samples. We then compared the proportion of organisms

detected in healthy infants with the proportion of mean detections in infants with *Ureaplasma* illness. Proportions were compared using the Z statistic, at alpha level=0.05.

To evaluate whether *Ureaplasma* illness could instead be attributed to other pathogen(s), we analyzed the subset of infants with an individual *Ureaplasma* probability >50%, i.e. a subset with a high probability of illness attributable to *Ureaplasma.* We compared the average individual probabilities for all organisms for this subset both with and without the *Ureaplasma* contribution to etiology included by removing the *Ureaplasma* probabilities and rescaling the remaining pathogen attribution probabilities to sum to 100%.

To describe the deaths among infants with *Ureaplasma*-attributable illness in this population, we compared the average individual *Ureaplasma* probabilities among cases who died and who survived, to ascertain if there was a higher median probability of *Ureaplasma* attribution among deaths.

To describe characteristics among *Ureaplasma* attributable population, [30] we calculated the posterior probability for each selected variable, based on the ANISA pLCM output, using the standard Bayesian formula: *P(variable|Ureaplasma etiology)=P(variable x Ureaplasma etiology)/P(variable).* We then used the posterior probabilities from every iteration to obtain a distribution for each variable; point estimates (means) and 95% Bayesian credible intervals (2.5th and 97.5th percentiles) were reported. We stratified these probabilities by early and late onset of disease, and separately by preterm and full-term gestational age. Credible intervals based on an alpha=0.05 that did not overlap were considered statistically significant.

Study power

We performed a power calculation for a case control analysis using logistic regression. Assuming a 5% alpha level, with ~240 cases and ~57,000 controls, and an average exposure proportion in the controls of 25%, we estimate our power to be 80% to detect an odds ratio of at

least as high as 1.5. If we varied the exposure proportions to 15%, 20%, and 40%, the corresponding power to detect an odds ratio of at least 1.5 is 70%, 77%, and 86%, respectively.

Study limitations

There are a high number of infants meeting the case definition from the etiology study that had a low probability (<10%) of their illness attributable to *Ureaplasma spp*. The results of the second analysis may have been strengthened if we had observed a larger number of infants with a higher probability of *Ureaplasma* spp. infection among our case population, allowing us to limit our analysis to these infants. Data collection was nearly 100% complete for all cases, but the level of missingness is higher among the larger cohort. The assessment of missingness is not complete for all variables of interest, we do know that the number of antenatal care visits (28%), first birth (13%), and materials used to cut and tie the umbilical cord (12%) have over 10% missing values.

Risks to Human Subjects

Human subjects involvement, characteristics and design

IRB approval for the ANISA study was obtained from each of the study sites' local IRBs in Bangladesh (International Centre for Diarrhoeal Disease Research, Bangladesh), India (Asian Institute of Public Health and Christian Medical College), and Pakistan (Aga Khan University), as well as partner institutions of Johns Hopkins University Bloomberg School of Public Health and the Hospital for SickKids. The Centers for Disease Control and Prevention relied on all local IRBs for ethical review. Consent was obtained from all mothers or caregivers who were enrolled in this study.

The study is a secondary analysis of the ANISA cohort. We used data previously collected on 63,114 infants and enrolled in the pregnancy and infant surveillance, which includes 5,253 infants that met the case definition, and 1895 healthy controls. Both study objectives are

included under the original written consent form obtained from the mother/caregiver at study enrollment. The study population will include mothers and their infants originally enrolled in the parent study. No new enrollment occurred study. Young infants are included as the study results will be directly beneficial for them and consent will be obtained from their parents/care givers.

Patient confidentiality

All the research documents and specimens are held confidential and only shared with individuals who are directly involved in the study. Study questionnaire and data forms are kept in a secured place and accessible only to study staff. The patient identifiers were separated from the coded questionnaires and kept in locked file at study sites. No personal identifiers were entered in the database, and no personally identifiable information was ever shared with CDC staff. Data confidentiality will be maintained at all times. No personal identifiers will be used in any reports or publications of study.

CHAPTER 4: INFECTIOUS ETIOLOGIES OF NEONATAL ILLNESS CLASSIFIED USING WORLD HEALTH ORGANIZATION DEFINITIONS

Results

There were 934 infants in the critically ill category, 3769 in the clinical severe infection category, and 738 in the late onset isolated fast breathing category. Infants meeting the critically ill case definition and the clinical severe infection case definition were more likely to have been born preterm or low birth weight when compared to late onset isolated fast breathing infants (Table 1). Critically ill infants were significantly more likely to be hospitalized (48.8%) or died within 7 days of illness onset (18.2%) when compared with those who met the clinical severe infection case definition (31.8% hospitalized, 3.7% died) and those with isolated fast breathing (14.5% hospitalized, 1.6% died). ANISA only captured clinical and diagnostic data on 9% of infants who died within 3 days of illness onset.[18] Among the 9% of deaths for which a physician assessment was performed, respiratory and blood specimens were collected from 9.5% of infants in the clinical severe infection group, 22.9% of those in the critically ill group, and 2.1% of those in the isolated fast breathing group.

Among those infants meeting the case definition for clinical severe infection, the leading pathogens identified were respiratory syncytial virus (RSV) (6.9%; 95%CI: 6.2%, 7.9%), *Ureaplasma* spp. (3.1%; 95%CI: 2.1%, 4.1%), and other bacterial pathogens not tested for by TAC (2.4%; 95%CI: 2.0%, 3.0%). A large proportion of the population with clinical severe infection (71.7%; 68.9, 74.5) did not have an etiologic attribution (Figure 5).

A comparison of episodes attributable to pathogens in the critically ill group versus the clinical severe infection revealed more illness attributable to bacteria in the critically ill (33%)

vs. clinical severe infection group (14%). Higher proportions of critically ill infants were attributed to other bacterial pathogens not tested for by TAC (7.9%; 95%CI: 6.2%, 9.8%), *Klebsiella pneumoniae* (5.6%; 95%CI: 3.2%, 8.3%), *E. coli* (3.9%; 95%CI: 2.5%, 5.8%) and *Ureaplasma s*pp. (5.5%; 95%CI: 3.6%, 7.7%). (Figure 2). A higher proportion of critically ill case episodes were attributed to any infectious etiology (41.8%; 95%CI: 36.3%, 48.2%) compared to clinical severe infection case episodes (27.1%; 95%CI: 24.3%, 30.4%).

Overall, 9% of late onset isolated fast breathing cases had an attributable bacterial etiology (Figure 6). RSV was the leading pathogen among infants with late onset isolated fast breathing (3.4%; 95%CI: 2.0%, 4.9%) and those with clinical severe infection (15.7%; 95%CI: 13.0%, 18.1%); however, the proportion of episodes attributable to RSV was much higher among infants with late-onset clinical severe infection. There were significantly higher proportions of *E. coli* (1.4% (95%CI: 0.6%, 2.7%) vs 0.1% (95%CI: 0.1%, 0.5%)) and Enterovirus/Rhinovirus (4.2% (95%CI: 2.4%, 6.5%) vs 0.8% (95%CI: 0.1%, 1.8%)) in the clinical severe infection group when compared with the late onset isolated fast breathing group. Although not significant, almost all of the pathogens had higher proportions in the clinical severe infection group, except for *Salmonella* and human metapneumovirus. A significantly higher proportion (82.5%; 95%CI: 78.7%, 86.0%) of the isolated fast breathing cases could not be attributed to any etiology, as compared with 51.3% (95%CI: 45.7, 59.9) of late-onset clinical severe infection cases.

A comparison of the clinical presentation of infants with clinical severe infection among those with a bacterial vs. viral etiology showed several significant differences in proportions of danger signs between the two groups (Table 2a). Those with bacterial etiology had significantly higher proportions of hypothermia (12.2% (95%CI: 10.9%, 13.4%) vs 3.7% (95%CI: 2.9%,

4.4%)), movement only when stimulated (18.6% (95%CI: 17.0%, 20.1%) vs 6.5% (95%CI: 5.5%, 7.6%)), convulsions (4.5% (95%CI: 4.0%, 5.1%) vs 1.5% (95%CI: 1.2%, 1.9%)), and poor feeding (47.9% (95%CI: 45.1%, 50.5%) vs 26.1% (95%CI: 24.3%, 28.0%)) when compared to those with a viral etiology. Infants with viral attribution had higher proportions of fast breathing (42.4% (95%CI: 41.2%, 43.6%) vs 37.3% (95%CI: 35.7%, 38.3%)) and severe chest in-drawing (51.1% (95%CI: 48.6%, 53.6%) vs 22.0% (95%CI: 19.8%, 24.3%)) when compared to those with a bacterial etiology. When further stratifying this comparison by early and late onset of illness, there were significant differences in clinical presentation between those infants with early and late onset of disease (Table 2b). Among infants with early onset of disease, there was a significantly higher proportion of infants with movement only when stimulated or no movement (24.2% (95%CI: 21.8%, 26.6%) vs 12.0% (95%ci: 6.2%, 18.2%)) and poor feeding (67.3% (95%CI: 63.8%, 70.2%) vs 54.7% (95%CI: 45.6%, 62.3%)) in the bacterial etiology group when compared to the viral group. Among infants with late onset disease, the bacterial etiology group had significantly higher proportions of every sign except for fast breathing and severe chest in drawing, which were higher in the viral etiology group. When comparing the bacterial etiology groups across early and late onset of disease, there were significantly higher proportions of every sign in the early onset group, except for severe chest in drawing and fever, which were significantly higher in the late onset group. When making the same comparison in the viral etiology group, there were significantly higher proportion of infants with hypothermia, convulsions, and poor feeding in the early onset group, while there were higher proportions of infants with severe chest in drawing in the late onset group.

Discussion

Limited etiologic evidence was available to inform the development of the original IMCI guidelines for management of illness in young infants; in particular, the studies available were

limited by small sample sizes and blood culture as the only diagnostic test.[26-28] The ANISA study is the first of its kind to characterize the etiologic spectrum in young infants presenting with pSBI in a large prospective multisite cohort study in settings where referral refusal is high. Infants meeting the critically ill case definition had higher proportions of bacterial infections (33%), which warrants continued reinforcement that referral is imperative for infants in this category.

The data for young infants with late onset isolated fast breathing generally support the revised guideline that recommends treatment of infants in this category with outpatient oral antibiotics. While 18% of pSBI cases in this group were attributed to any infectious etiology, half of these were attributed to viral infection.

Most notably, while the etiologic data support recent WHO distinctions in clinical presentation, there is still a large proportion of unexplained illness in all three groups of infants, despite testing for 28 pathogen classes. While it is possible that specimens obtained in ANISA were not adequate to detect all infections (e.g., no collection of cerebrospinal fluid or urine), or a pathogen contributing to the unexplained etiology group was not included in the test panel (i.e. the diagnostic methods used were not able to identify all potential etiologies), it is also plausible that many episodes of pSBI are not caused by acute infection.

Given that the majority of young infants in the ANISA cohort had an unexplained illness etiology, we further examined the clinical presentation of infants meeting the clinical severe infection definition in an effort to identify clinical signs associated with bacterial as opposed to viral infections. Infants in the clinical severe infection group with a bacterial etiology had significantly higher proportions of the clinical signs typically associated with more severe infection (such as hypothermia, convulsions and movement only with stimulation). Although

there were some individual clinical signs that were more prevalent among infants with bacterial versus viral etiologies or vice versa, there is no evidence that individual signs or groups of signs can be used to predict bacterial versus viral etiology. While recent research suggests that biomarkers such as C-reactive protein and certain toll-like receptors[29], as well as new meta-genomic sequencing to rapidly identify pathogens from normally sterile fluids, may help diagnose infection, it is unlikely that physicians would have this information or technology available in low resource public health settings. In light of these data, these data do not provide supportive evidence for further refinements to the case definition to distinguish bacterial from viral infection based on clinical signs.

While the proportion of infants with a bacterial etiology was highest in the most severe category, and lowest in the least severe category, there was still a non-zero proportion of those with bacterial etiology in every category. If the trials conducted in Africa and South Asia that evaluated the equivalency of simpler antibiotic regimens to the conventional regimens [14-16] had limited their study population to those with a bacterial etiology, simplified regimens may not have performed as well. However, in the absence of a method available to identify etiology of illness more readily in low resource settings, this analysis supports the current set of case definitions and the 2015 WHO guideline as appropriate tools for referral and treatment among those refusing referral.

Lastly, although currently recommended treatment regimens target most of the bacteria identified in infants in all three illness categories defined by WHO; *Ureaplasma* represents a comparatively high proportion of infection in all groups. *Ureaplasma* has not been identified previously as a relevant pathogen for this age group, and the currently recommended regimens (typically gentamicin or amoxicillin) would not be effective against this agent. Regimens used to

treat *Ureaplasma* infection typically include macrolides or tetracyclines. [30] In the most severely ill infants in the ANISA population, 5.5% of episodes would not have received antibiotic therapy effective against the etiologic agent of illness.

While some analyses presented here have a small sample size, such as the comparison of etiologic proportions among infants with late onset isolated fast breathing vs. infants with clinical severe infection, these are the first data that describe the etiology of infections in young infants meeting all three WHO case definitions. Ascribing etiologic attribution is challenging, due to the fact that every infant has multiple results for many pathogens. While the pLCM method allows for use of multiple test results to be used in the estimation of etiology proportions, there are certain circumstances and conditions when using the pLCM methods, such as low number of positive detections, or having only a single test available for a given pathogen, where estimation of the true etiology proportion has limited reliability. These are important aspects of our study to consider when interpreting the results. A series of simulations to validate this model identified mitigation strategies for the scenarios in which we have single tests available or low number of positive detections, such as inclusion of pathogens which have multiple tests and high number of detections, and subjecting pathogens with a single test or low numbers of detections to a rigorous selection process.[23] As in the parent study, we incorporated these strategies, hence we believe the model results were reliable for our analysis.

The ANISA study was conducted in countries with relatively high burden of neonatal disease and mortality, and high rates of refusal of referral for treatment/hospitalization, thereby providing the appropriate population to describe vital data on the etiologic spectrum of disease in young infants. The data suggest it would be difficult to develop a clinical algorithm to distinguish bacterial from viral infections. Furthermore, none of the case definitions evaluated

here distinguish well between infectious and non-infectious causes of illness. While infants are likely being unnecessarily treated with antibiotics in these settings, given they meet the case definition but may have a viral etiology or no infectious etiology at all, there are infectious etiologies found even among the least severely ill infants. These data suggest that there may be a substantial proportion of noninfectious illness for which other interventions would be more effective.

Table 1. Characteristics of infants presenting with possible serious bacterial infection 11, stratified by World Health Organization case definition, Aetiology of Neonatal Infections in South Asia (ANISA) Study, 2011-14

Characteristic (% unless otherwise specified)	Critically ill N (%)	Clinical severe infection N (%)	Isolated Fast breathing, 3-59 days N (%)
Characteristic (% unless otherwise specified)			
Infant	934	3769	738
Male	545 (58.3)	2114 (56.1)	441 (59.8)
Preterm	268 (28.7)	1050 (27.9)	132 (17.9)
Low birthweight	357 (38.2)	1373 (36.4)	185 (25.1)
pSBI episodes	992	4000	771
Early-onset[1]	508 (51.2)	2448 (61.2)	0
Integrated Management of Childhood Infection Signs			
Respiratory rate >=60 breaths per minute	375 (37.8)	1580 (39.5)	771 (100)
Severe chest in-drawing	153 (15.4)	1086 (27.2)	0
Axillary temperature =>38.0`C (>100.4`F)	229 (23.1)	1608 (40.2)	0
Axillary temperature <35.5`C (<95.9`F)	157 (15.8)	377 (9.4)	0
Movement only when stimulated	307 (31.0)	332 (8.3)	0
No movement at all (unconscious)	44 (4.4)	0	0
Convulsions (confirmed by observation)	223 (22.5)	0	0
Poor feeding (confirmed by observation)	680 (68.6)	1455 (36.4)	0
Other Signs			
Bulging fontanelle	40 (4.0)	0	0
Persistent vomiting	64 (6.5)	0	0
Unable to cry	306 (30.9)	0	0
Presence of apnea	92 (9.3)	0	0
Presence of cyanosis	314 (31.7)	0	0
Child Hospitalized	484 (48.8)	1562 (31.8)	112 (14.5)
Child Died[2]	227 (22.9)	251 (9.5)	16 (2.1)
Child Died within 7 days of episode[2]	181 (18.2)	146 (3.7)	12 (1.6)

[1] Early onset is defined as < 3 days of life

[2] Deaths with clinical information available are presented.

Table 2. Distribution of signs among infants meeting the clinical severe infection case definition (n=4000), by viral or bacterial etiology, Aetiology of Neonatal Infections in South Asia (ANISA) Study, 2011-14

Characteristic (% unless otherwise specified)	Bacterial etiology % (95%CI)	Viral etiology % (95%CI)
Characteristic (% unless otherwise specified)		
Respiratory rate >=60 breaths per minute	37.3 (35.7,38.8)	42.4 (41.2, 43.6)
Severe chest in-drawing	22.0 (19.8,24.3)	51.1 (48.6, 53.6)
Axillary temperature >38.0`C (>100.4`F)	32.9 (31.2, 34.9)	32.8 (31.1, 34.4)
Axillary temperature <35.5`C (<95.9`F)	12.2 (10.9, 13.4)	3.7 (2.9, 4.4)
Movement only when stimulated or no movement	18.6 (17.0, 20.1)	6.5 (5.5, 7.6)
Convulsions (confirmed by observation)	4.5 (4.0,5.1)	1.5 (1.2, 1.9)
Poor feeding (confirmed by observation)	47.9 (45.1, 50.5)	26.1 (24.3, 28,0)

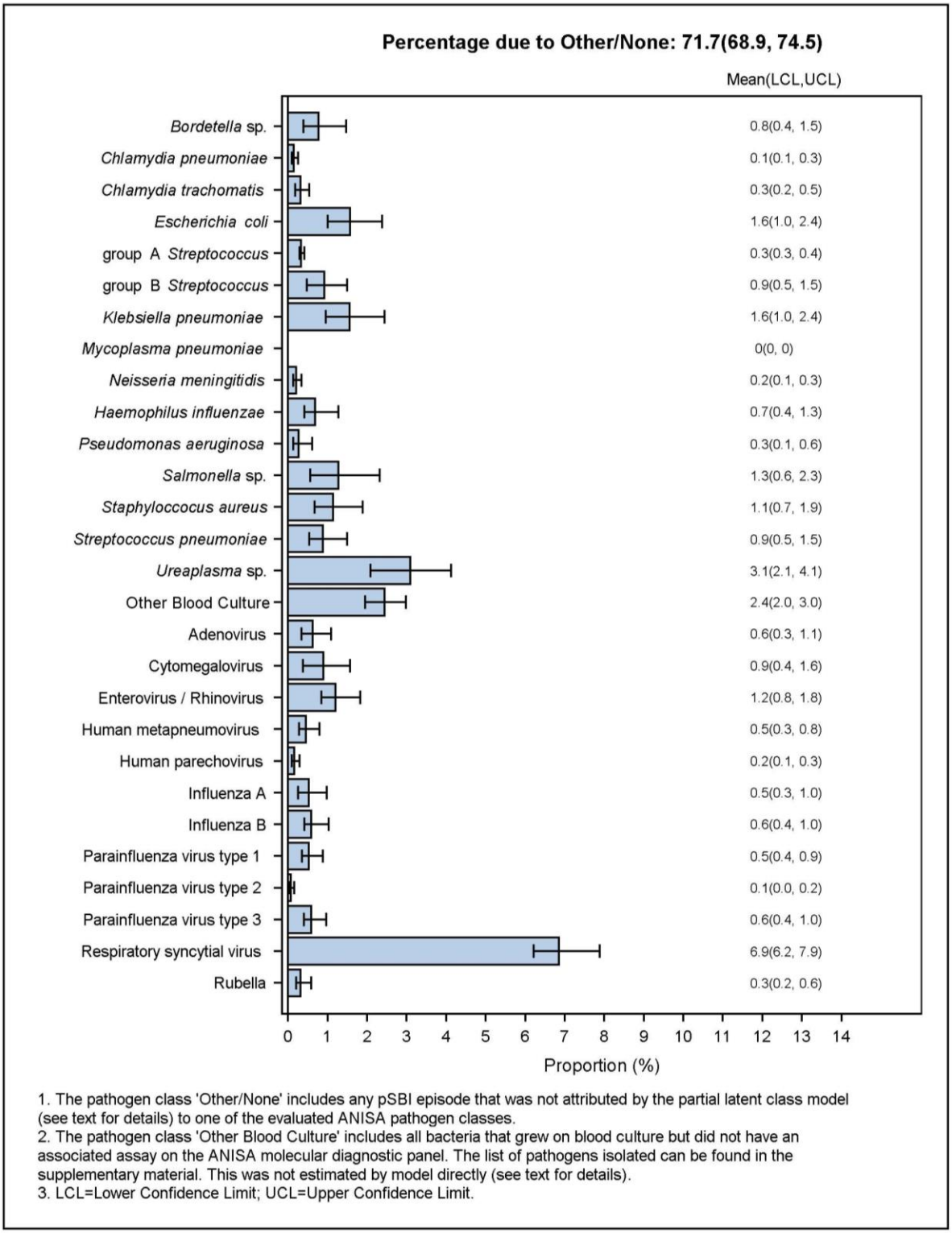
[1] Early onset is defined as < 3 days of life
[2] Late onset is defined as => 3 days and <60 days of life
[3] Temperature was obtained in F, but reported in C after conversion

Table 3. Distribution of signs among infants meeting the clinical severe infection case definition (n=4000), by age of onset and bacterial and viral etiology, Aetiology of Neonatal Infections in South Asia (ANISA) Study, 2011-14

Characteristic (% unless otherwise specified)	Early onset[1] % (95%CI)		Late onset[2] % (95%CI)	
Characteristic (% unless otherwise specified)				
	Bacterial	Viral	Bacterial	Viral
Respiratory rate >=60 breaths per minute	41.8 (39.7, 43.8)	38.4 (32.2, 44.4)	34.5 (32.6, 36.5)	42.7 (41.4, 44.0)
Severe chest in-drawing	9.6 (8.5, 10.7)	5.7 (3.1, 9.7)	29.6 (26.7, 32.6)	54.2 (51.9, 56.3)
Axillary temperature >38.0`C (>100.4`F)	26.1 (23.3, 29.4)	29.5 (21.0, 40.5)	37.1 (35.2, 38.9)	33.0 (31.5, 34.5)
Axillary temperature <35.5`C (<95.9`F)	19.8 (17.8, 21.8)	24.0 (17.7, 30.2)	7.5 (6.4, 8.5)	2.3 (1.9, 2.7)
Movement only when stimulated or no movement	24.2 (21.8, 26.6)	12.0 (6.2, 18.2)	15.2 (13.7, 16.7)	6.1 (5.2, 7.1)
Convulsions (confirmed by observation)	8.4 (7.3, 9.5)	5.6 (2.3, 9.2)	2.1 (1.8, 2.5)	1.2 (1.0, 1.4)
Poor feeding (confirmed by observation)	67.3 (63.8, 70.2)	54.7 (45.6, 62.3)	36.0 (33.2, 38.9)	24.2 (22.4, 25.9)

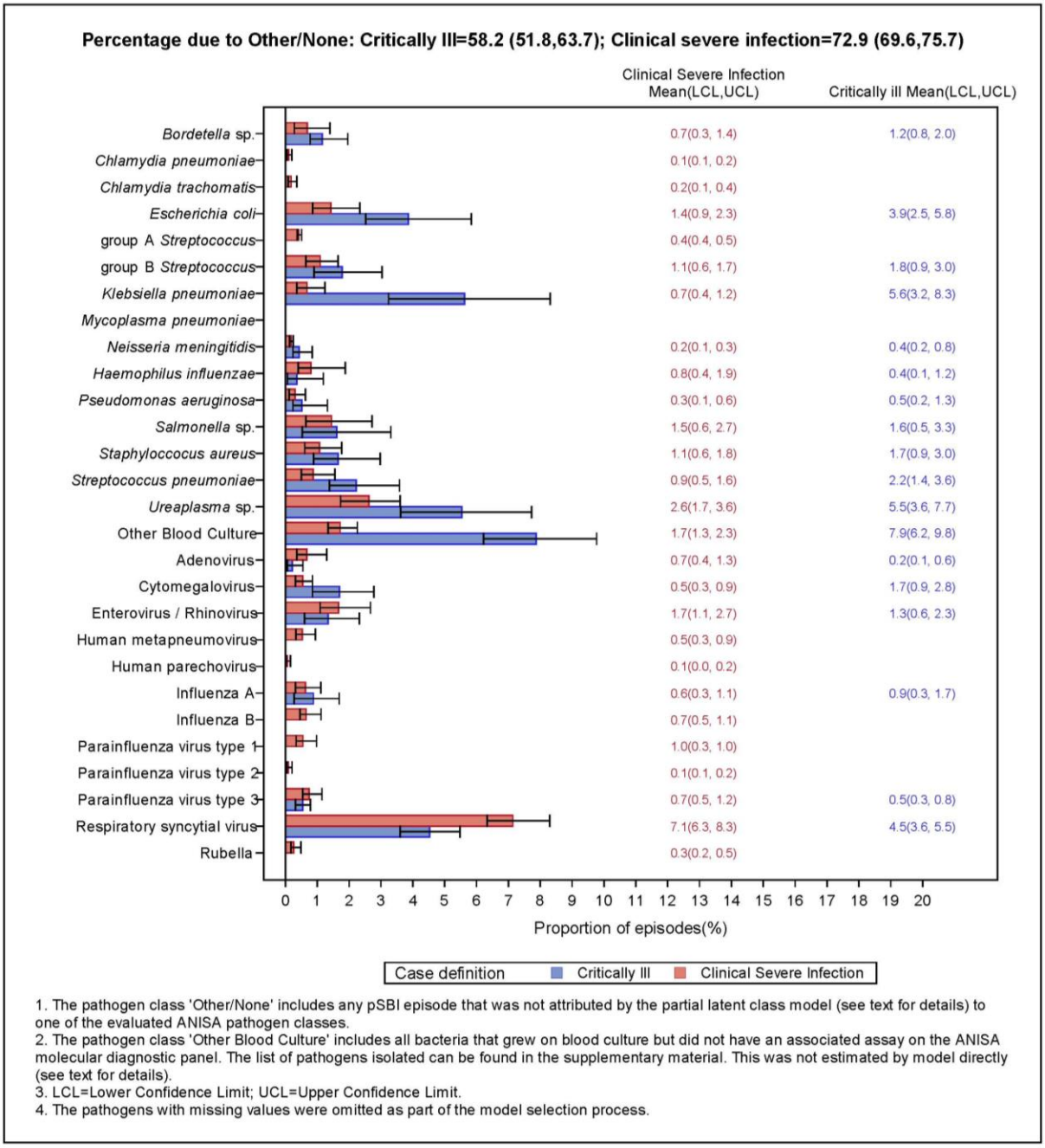
[1] Early onset is defined as < 3 days of life
[2] Late onset is defined as => 3 days and <60 days of life
[3] Temperature was obtained in F, but reported in C after conversion

Figure 5. Estimates from a partial latent class model of the prevalence of pathogens in infants meeting clinical severe infection case definition (N=4000), Aetiology of Neonatal Infections in South Asia (ANISA) Study, 2011-14



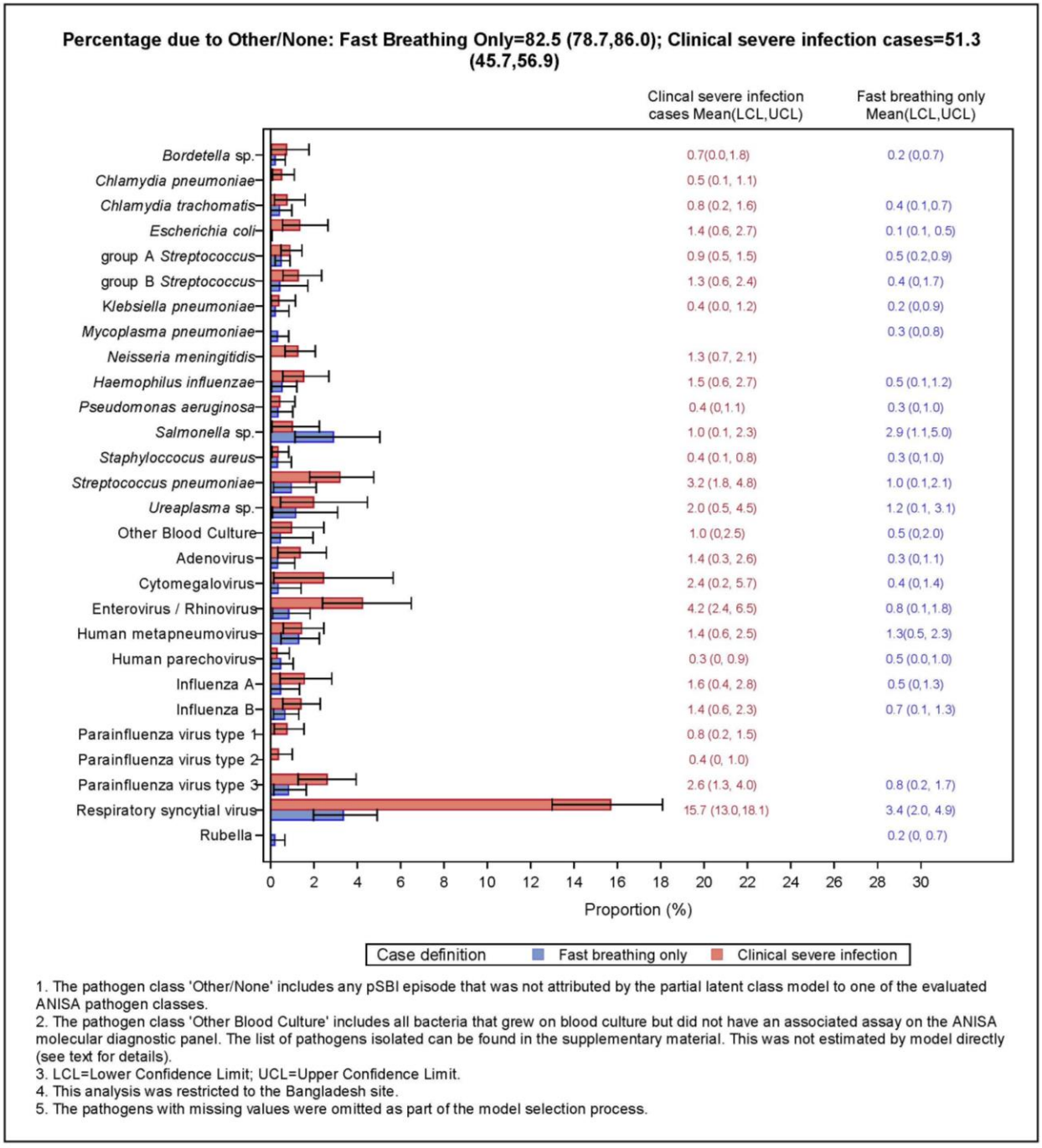
1. The pathogen class 'Other/None' includes any pSBI episode that was not attributed by the partial latent class model (see text for details) to one of the evaluated ANISA pathogen classes.
2. The pathogen class 'Other Blood Culture' includes all bacteria that grew on blood culture but did not have an associated assay on the ANISA molecular diagnostic panel. The list of pathogens isolated can be found in the supplementary material. This was not estimated by model directly (see text for details).
3. LCL=Lower Confidence Limit; UCL=Upper Confidence Limit.

Figure 6. Estimates from a partial latent class model of the prevalence of pathogens in infants meeting the critically ill case definition (n=992) compared to those meeting the clinical severe infection case definition (n=4000), Aetiology of Neonatal Infections



1. The pathogen class 'Other/None' includes any pSBI episode that was not attributed by the partial latent class model (see text for details) to one of the evaluated ANISA pathogen classes.
2. The pathogen class 'Other Blood Culture' includes all bacteria that grew on blood culture but did not have an associated assay on the ANISA molecular diagnostic panel. The list of pathogens isolated can be found in the supplementary material. This was not estimated by model directly (see text for details).
3. LCL=Lower Confidence Limit; UCL=Upper Confidence Limit.
4. The pathogens with missing values were omitted as part of the model selection process.

Figure 7. Estimates from a partial latent class model of the prevalence of pathogens in infants presenting with isolated fast breathing (n=771), compared to those meeting the clinical severe infection case definition (n=1552), late onset of illness (=>3 - 59 day



1. The pathogen class 'Other/None' includes any pSBI episode that was not attributed by the partial latent class model to one of the evaluated ANISA pathogen classes.
2. The pathogen class 'Other Blood Culture' includes all bacteria that grew on blood culture but did not have an associated assay on the ANISA molecular diagnostic panel. The list of pathogens isolated can be found in the supplementary material. This was not estimated by model directly (see text for details).
3. LCL=Lower Confidence Limit; UCL=Upper Confidence Limit.
4. This analysis was restricted to the Bangladesh site.
5. The pathogens with missing values were omitted as part of the model selection process.

CHAPTER 5: CHARACTERIZING NEONATES WITH ILLNESS ATTRIBUTABLE TO *UREAPLASMA* SPP. INFECTIONS

Results

Artifacts of assays

All bacteria, including *Ureaplasma*, with at least two molecular tests available (Table 4) were not frequently detected in both blood and respiratory specimens, i.e. concordance between respiratory and blood detections was low. Six (19%) of the 31 blood specimens positive for *Ureaplasma* were also positive on respiratory TAC, and 1% of respiratory specimens were also positive on blood TAC. Among those speciated (n=498), 57% were *U. parvum*, 31% were *U. urealyticum*, 4% were both species, and 8% could not be speciated.

Observed diagnostic results

Ureaplasma spp. were detected in higher numbers in infants with suspected infection compared to healthy infants. Crude odds ratios (cORs) varied by specimen type and study site, ranging from 1.5 to 2.7. When stratified by age at onset of disease, cORs were higher for infants with early onset disease. Among infants who died, cORs were 3.7 for respiratory specimens and 1.4 for blood specimens. Furthermore, the *Ureaplasma* detections were not limited to preterm infants. The cORs for *Ureaplasma* was higher among term infants than preterm infants (1.8 vs. 1.6 in respiratory specimens; 1.5 and 1.3 for blood specimens).[7]

Criteria for which ANISA modeled results are less reliable

Ureaplasma did not fall into any of the documented pLCM "danger zones". *Ureaplasma* was included in both the respiratory and blood molecular PCR TAC, so there were two test results available for every case. The overall cOR was > 1 for respiratory specimens in all study

sites, and >1 in 3 of the 5 study sites for blood specimens (the two Indian sites had cOR <1 in blood). The *Ureaplasma* population proportion was 2.8% (95% CI: 1.9%, 3.8%), the second highest in the study after RSV. The mean false positive rates (FPRs) ranged from 3.1% (95% CI: 2.1%, 4.21%) in Matiari to 12.6% (95% CI: 11.2%, 13.8%) in Odisha for respiratory specimens, and from 0.1% (95% CI: 0.1%, 0.23%) in Karachi to 0.4% (95% CI: 0.2%, 0.6%) in Sylhet for blood specimens. [7] The specificity was low and similar to those for other pathogens. Sensitivity (constant across sites) was 61.5% (95%CI: 48.3%, 76.2%) for the respiratory molecular test and 22.2% (95%CI: 20.1%, 27.6%) for the blood molecular assay, both above all specificity estimates.

Ureaplasma as commensal organism vs pathogen causing illness

Overall, 26% of healthy infants and 28% of *Ureaplasma* attributable cases had nothing other than *Ureaplasma* detected in either blood or respiratory specimens (Table 5). A few pathogens (group A *Streptoccocus* and *Mycoplasma pneumoniae*, and viruses influenza B, parainfluenza virus 3, and RSV) were more common in *Ureaplasma* attributable infants vs healthy infants, but the differences in proportions were small. .

Based on the ANISA pLCM results, 68 cases had an average individual *Ureaplasma* probability > 50% (Table 6). In this subset, 73.7% (95%CI: 51.9%, 100.0%) of infants had illness attributable to *Ureaplasma*, and 22.2% (95%CI: 0%, 46.7%) had no identifiable infectious etiology. Only *E. coli* had an average individual probability of 1% or greater in this population. After removing the *Ureaplasma* etiologic contribution in this subset, 83.3% (95%CI: 13.1%, 97.0%) of the population had no infectious etiology identified;85% of the *Ureaplasma* contribution did not assign to any other pathogen once set to zero, while the remaining 15% of the individual case probabilities were re-distributed among *E. coli* (3.1% (95%CI: 0,57%.7%)),

*Streptococcus pneumoniae (*2.6% (95%CI: 0,66%.9%)), other bacteria identified by blood culture (2.4% (95%CI: 0%,15.3%)), *Klebsiella pneumoniae (*1.7% (95%CI: 0%,44.8%)), Cytomegalovirus (1.7% (95%CI: 0%,61.7%), and *Bordetella* sp. (1.3% (95%CI: 0%,80.9%). After *Ureaplasma*'s contribution was removed from this population, *Bordetella* sp., *E. coli*, *Streptococcus pneumoniae*, and Cytomegalovirus average individual probabilities were higher than the population probabilities estimated in the primary etiology study, and of these, *E. coli*, *Streptococcus pneumoniae*, and Cytomegalovirus averages were higher than the upper credible interval limit of these estimated probabilities.

Describing deaths among infants with Ureaplasma-attributable illness

The overall distribution of average individual *Ureaplasma* probabilities among those who survived is more heavily right skewed (skewness=2.09) than for those who died (skewness=1.19) (Figure 8). The median individual *Ureaplasma* probability was 2 times as high among infants who died (15%) when compared to cases who survived (7%) (p-value <0.0001). Among infants with *Ureaplasma* attributable illness, 27.2% (95%CI: 21.2%, 34.5%) were hospitalized, and 22.7% (95%CI: 15.5%, 30.3%) died (Tables 7 and 8). In comparison, 29% of infants who met the ANISA case definition were hospitalized, and 13% of them died.[7]

Characteristics of infants with Ureaplasma attributable illness

Over a third (38.3%) of mothers with infants who were among the *Ureaplasma* attributable population, had attended school at any time, and 3.8% were under the age of 20 years at the time of delivery (Tables 7 and 8). The proportion of mothers with at least 1 antenatal care visit was 77.8% (95%CI: 73.4%, 81.3%), 44.6% (95%CI: 38.9%, 50.9%) received the full antenatal package, and 9.1% (95%CI: 7.4%, 10.6%) had measured poor nutritional status. Few mothers died (0.02% (95%CI: 0.0%, 0.04%)).

Among infants with *Ureaplasma* attributable illness, 57.2% (95%CI: 54.3%, 60.0%) were male, 22.0% (95%CI: 19.2%, 24.8%) were preterm, and 50.0% (95%CI: 46.1%, 53.7%) of them were low birthweight. Among infected infants, the most common danger signs observed were poor feeding (39.8% (95%CI: 33.9%, 46.0%)), fast breathing (39.1% (95%CI: 35.7%, 42.2%)), and fever (34.1% (95%CI: 29.1%, 38.6%)).

Infants with early onset illness had significantly higher proportions of complications or illness since birth (50.2% (95%CI: 46.7%, 54.0%)) when compared with infants with late onset illness 20.7% (95%CI: 17.3%, 24.3%) (Table 4a). The only significant difference in clinical danger signs was the higher proportion of preterm infants with poor feeding (53.1% (95%CI: 44.5%, 61.9%)) when compared to term infants (36.8% (95%CI: 30.8%, 43.0%)). As expected, no infant in the *Ureaplasma* attributable population was treated with appropriate antibiotics. Of all of the hospitalization episodes recorded in the ANISA cohort (n=2344), tetracycline and doxycycline were never prescribed, and erythromycin, clindamycin, and clarithromycin were prescribed less than twice each. Azithromycin was prescribed less than 2% of the time (n=43).

Discussion

We were unable to identify a viable alternative explanation for the ANISA *Ureaplasma* finding. Furthermore, ANISA describes the infant population with *Ureaplasma* attributable illness as having different clinical characteristics than what has previously been described in the literature. ANISA is one of the first studies of its kind to implement molecular diagnostics in addition to traditional blood culture methods, and to employ modeling strategies incorporating all diagnostic evidence to improve estimates of neonatal illness etiology. Given the diagnostic challenges with growing *Ureaplasma* on culture and lack of access to advance molecular

diagnostic tools, this has likely led to *Ureaplasma* previously being underdetected in the clinical setting.

Among respiratory specimens (the source of the majority of *Ureaplasma* detections), *Ureaplasma* detections were more common among cases than healthy infants in every study site. The differences in *Ureaplasma* detections between sick and healthy infants was even higher among term infants than in preterm infants, whereas *Ureaplasma* has been described in the literature as being an infection in preterm infants. Importantly, 93% of all respiratory or blood specimens positive for *Ureaplasma* in the ANISA study were speciated to either *U. parvum, U. urealyticum,* or both, confirming that the diagnostic results were not an artifact of the TAC assays. While concordance was low between blood and respiratory specimens, the lower concordance would only drive the population proportion lower, not higher, than other pathogens with higher concordance.

The proportions of detections of other bacteria and viruses between healthy infants and *Ureaplasma* attributable cases were similar, with few significant differences for a handful of organisms. The proportion of infants with nothing other than *Ureaplasma* identified were also similar between the two groups. Taking a step further and examining illness attribution using the ANISA pLCM model without *Ureaplasma*'s contribution in the population, the majority (85%) of *Ureaplasma*'s contribution was re-distributed to the "no identifiable infectious etiology" category, with the rest of *Ureaplasma*'s contribution re-distributed to a handful of bacteria. Only *E. coli, S. pneumoniae* and Cytomegalovirus average probabilities increased above the upper confidence limits estimated in the primary ANISA etiology study. These data suggest there was no other organism was co-detected frequently enough, or whose attribution proportion rose

sharply once *Ureaplasma* was removed from the population, to suggest that *Ureaplasma* was a commensal organism found in conjunction with another pathogen causing disease.

The description of the infant population with *Ureaplasma* attributed illness highlighted how different this population is from infants with *Ureaplasma* illness described previously in the literature. The literature describes extremely and very preterm infants with *Ureaplasma* infection, with clinical presentation manifesting as bronchopulmonary dysplasia, sepsis, and mild cerebral impairment. There were similar numbers of preterm infants with *Ureaplasma* attributable illness (22% vs 20%) when compared to the entire enrolled population, and less than 3% of them were extremely or very preterm. A higher proportion of infants with *Ureaplasma* attributable illness (24%) died when compared with all infants who met the case definition (13%), and the median average individual *Ureaplasma* probability was 2 times as high among deaths (15%) when compared to cases who survived (7%). These findings suggest that *Ureaplasma* causes severe disease, or infants with *Ureaplasma* attributable illness had more severe outcomes due to treatment with antibiotics that are not effective against this agent, or both.

While there have been several studies describing the relationship between *Ureaplasma* colonization (detection in asymptomatic individuals) and adverse maternal and neonatal outcomes, these findings have been limited to preterm infants. [31-33] Only one study has found placental infection with *Ureaplasma* spp. associated with chorioamnionitis regardless of gestational age, and further adverse outcomes (requiring oxygen or positive pressure respiratory support for prolonged periods) in those infants that were moderate to late preterm. [34] There is one case report in the literature that describes late onset meningitis in a full term newborn caused by *Ureaplasma parvum* in the United States.[35]

A recent etiology study conducted in Soweto, South Africa, using a very similar protocol and diagnostic methods to the ANISA study, reported *Ureaplasma* as the most important pathogen causing neonatal illness in their study population. They reported a *Ureaplasma* incidence rate of 2.11 per 1,000 live births, which represents 5.4% of the study population were *Ureaplasma* attributable cases. Notably, over half (52.7%) of the infants in the study population were term (=>37 weeks).

There were limitations to our study. As in the primary ANISA etiology study, the ANISA case definition is very sensitive and not specific, potentially leading to misclassification of infants. Blood culture was the 'gold' standard within the ANISA study—having a blood culture positive increased the probability of attribution to that pathogen. As *Ureaplasma* does not grow in standard blood culture media, there were no *Ureaplasma* isolated from blood culture in this study. The lack of blood culture results is one possible theory for the vast majority of the individual probabilities for *Ureaplasma* etiology being less than 10%, despite crude ORs above 1. Even with this limitation, *Ureaplasma* was the second highest estimated population proportion in the study. While some analyses presented here have a small sample size, these are the first data that describe term infants with *Ureaplasma* attributable illness.

Ureaplasma has not been identified previously as a relevant pathogen for young infants, and the current WHO recommended regimens (typically gentamicin or amoxicillin) would not be effective against this agent. Any future studies investigating *Ureaplasma* as a causative agent of illness in neonates, as well as the relationship between the presence of *Ureaplasma* with preterm and low birthweight infants, should use diagnostics that will allow for the detection of *Ureaplasma*. Further research would inform whether changes to current antibiotic regimens recommended by WHO for treatment of ill neonates are needed.

Table 4. Distribution of detectionsin blood and respiratory specimens tested by TaqMan Array (TAC) for bacteria with assays on both cards, Aetiology of Neonatal Infections in South Asia (ANISA) Study, 2011-2014

Bacteria	Total number of infants with detections in respiratory specimens	Total number of infants with detections in blood specimens	Number of infants with detections in both blood and respiratory specimens	% infants with detections in blood that also had detections in respiratory specimens	% infants with detections in respiratory specimens that also had detections in blood
group *B Streptoccocus*	550	25	10	29%	2%
Escherichia coli	1824	107	42	28%	2%
Klebsiella pneumoniae	1571	110	42	28%	3%
Streptococcus pneumoniae	2471	81	54	40%	2%
Ureaplasma sp.	684	25	6	19%	1%

[1] A positive detection in a sick infant is defined by a positive result on a TAC card from either respiratory or blood specimens. Group B *Streptococcus, Eschericia coli, Klebsiella pneumoniae, Streptococcus pneumoniae*, and *Ureaplasma* sp. assays were included on both the respiratory and blood TACs.

Table 5. Pathogen detections by TaqMan PCR in either blood or respiratory specimens1 in healthy infants and *Ureaplasma* attributable cases, Aetiology of Neonatal Infections in South Asia (ANISA) Study, 2011-2014

	Number of detections among healthy infants [2] N=1894 # (%)	Mean number of detections among cases with *Ureaplasma* attributable cases [3,4] mean N=155 # (%)	Z-test p-value
None [5]	496 (26)	43 (28)	0.67
Bordetella spp.	73 (4)	7 (4)	0.68
Chlamydia trachomatis	5 (0.3)	1 (1)	0.40
Chlamydia pneumoniae	3 (0.2)	1 (1)	0.19
group A *Streptococcus*	0	1 (1)	0.0004
group B *Streptococcus*	126 (7)	13 (8)	0.41
Eschericia coli	482 (26)	39 (26)	0.94
Haemophilus influenzae	6 (0.3)	2 (1)	0.06
Klebsiella pneumoniae	444 (23)	33 (22)	0.54
Mycoplasma pneumoniae	1 (0.1)	1 (1)	0.02
Neisseria meningitidis	4 (0.2)	1 (1)	0.29
Pseudomonas aeruginosa	7 (0.4)	1 (1)	0.60
Streptococcus pneumoniae	638 (34)	55 (35)	0.64
Salmonella spp.	30 (2)	3 (2)	0.74
Staphyloccocus aureus	15 (0.8)	2 (1)	0.51
Adenovirus	34 (2)	2 (1)	0.65
Cytomegalovirus	153 (8)	12 (8)	0.88
Influenza A	13 (0.7)	2 (1)	0.40
Influenza B	3 (0.2)	2 (1)	0.006
Human metapneumovirus	6 (0.3)	1 (1)	0.50
Human parechovirus	12 (0.6)	2 (1)	0.34
Parainfluenza virus 1	7 (0.4)	2 (1)	0.09
Parainfluenza virus 2	16 (0.8)	1 (1)	0.79
Parainfluenza virus 3	0	3 (2)	< .00001
Respiratory synctitial virus	25 (1)	12 (8)	< .00001
Rhinovirus/Enterovirus	644 (34)	45 (29)	0.21
Rubella	5 (0.3)	2 (1)	0.03

[1] A positive detection in a case is defined by a positive result on a TAC card from either respiratory or blood specimens. Adenovirus, *Bordetella sp.*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, Cytomegalovirus, Human metapneumovirus, Human parechovirus, Influenza A, Influenza B, *Mycoplasma pneumoniae*, Parainfluenza virus 1, Parainfluenza virus 2, Parainfluenza virus 3, Respiratory synctitial virus (RSV), Rhinovirus/Enterovirus, and Rubella assays were included on the respiratory TAC respiratory only. group A *Streptococcus*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Salmonella* spp., and *Staphyloccocus aureus* assays were included on the blood TAC only. group B *Streptococcus*, *Eschericia coli*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, and *Ureaplasma* spp. assays were included on both the respiratory and blood TACs.

[2] A healthy infant is an infant with clinical specimens and results who did not meet the case definition at the time of specimen collection.

[3] An ANISA case is defined as infant between 0 and 59 days of age that presented with one of the following signs, as confirmed by a physician: respiratory rate >=60/minute (fast breathing), severe chest in-drawing, axillary temperature either =>38°C (fever) or <35.5°C (hypothermia), movement only when stimulated or no movement at all, convulsions, or not feeding well confirmed on observation by study personnel.

[4] *Ureaplasma* attributable cases is defined as an individual infant who met the ANISA case definition, with a probabilistic assignment of *Ureaplasma* as the causative agent of illness, based on the estimated partial latent class model(pLCM) results.

[5] None is defined as having no other detections in either respiratory or blood specimens other than *Ureaplasma sp.*

Table 6. Distribution of average individual pathogen attribution probabilities for infant cases[1] both with and without *Ureaplasma* included, limited to infant cases with an average individual *Ureaplasma* probability >50%, Aetiology of Neonatal Infections in South Asia (ANISA) study, 2011-2014

	Average individual probabilities[2] N=68 % (95% CI)	Rescaled average individual probabilities[2] with *Ureaplasma* removed N=68 % (95% CI)
Bordetella sp.	0.1 (0,6.8)	1.3 (0,80.9)
Chlamydia pneumoniae	0 (0,0)	0 (0,0.5)
Chlamydia trachomatis	0 (0,0.1)	0.1 (0,0.5)
Escherichia coli	1.1 (0,2.6)	3.1 (0,57.7)
Group A *Streptococcus*	0 (0,0.2)	0 (0,0)
Group B *Streptococcus*	0.1 (0,0.6)	0 (0,1.6)
Klebsiella pneumoniae	0.6 (0,17.9)	1.7 (0,44.8)
Mycoplasma pneumoniae	0 (0,0)	0 (0,0)
Neisseria meningitidis	0 (0,0.1)	0 (0,0.6)
Haemophilus influenzae	0.1(0,0.8)	0 (0,1.9)
Pseudomonas aeruginosa	0.1 (0,1.5)	0 (0,3.8)
Salmonella sp.	0.2 (0,2.1)	0.7 (0,6.8)
Staphyloccocus aureus	0.2 (0,1.0)	0.8 (0,2.9)
Streptococcus pneumoniae	0.5 (0,10.8)	2.6 (0,66.9)
Ureaplasma sp.	73.7 (51.9,100.0)	0 (0,0)
Other Blood Culture	0.7 (0,6.5)	2.4 (0,15.3)
Adenovirus	0 (0,0.1)	0.1 (0,2.5)
Cytomegalovirus	0.2 (0,7.1)	1.7 (0,61.7)
Enterovirus / Rhinovirus	0.2 (0,3.3)	0.8 (0,12.1)
Human metapneumovirus	0 (0,0.8)	0.1 (0,4.0)
Human parechovirus	0 (0,0)	0 (0,0)
Influenza A	0.0 (0.0,0.0)	0 (0,1.7)
Influenza B	0 (0,0.5)	0.1 (0,1.4)
Parainfluenza virus type 1	0 (0,0.6)	0.2 (0,2.5)
Parainfluenza virus type 2	0 (0,0.1)	0 (0,0.3)
Parainfluenza virus type 3	0 (0,0.2)	0.1 (0,4.3)
Respiratory syncytial virus	0 (0,0.9)	0.2 (0,4.4)
Rubella	0 (0,0.1)	0 (0,0.4)
No etiology identified	22.2 (0,46.7)	83.3(0,97.4)

[1]An ANISA case is defined as infant between 0 and 59 days of age that presented with one of the following signs, as confirmed by a physician: respiratory rate >=60/minute (fast breathing), severe chest in-drawing, axillary temperature either =>38°C (fever) or <35.5°C (hypothermia), movement only when stimulated or no movement at all, convulsions, or not feeding well confirmed on observation by study personnel.

[2] An average individual probability is the likelihood from the ANISA pLCM model that a given organism is the causative agent of illness for a given case.

Table 7. Demographic and clinical characteristics of young *Ureaplasma* attributable cases[1], stratified by early and late onset of disease, Aetiology of neonatal Infections in South Asia (ANISA) Study, 2011-14

Characteristic	Early-onset[2] % (LCL,UCL)[3] N=2100	Late-onset %(LCL,UCL) N=3153	Overall % (LCL,UCL) N=5253
Characteristic			
Maternal			
First live birth	29.3 (22.6, 35.9)	29.7 (25.4, 34.6)	29.5 (25.0, 34.4)
Ever attended school	34.3 (24.0, 45.8)	41.5 (33.6, 50.4)	38.3 (30.2, 47.2)
Maternal age <20 years at delivery	4.4 (1.9, 7.3)	3.3 (2.1, 4.9)	3.8 (2.3, 5.7)
Pregnancy			
High grade fever during pregnancy	22.6 (17.7, 27.4)	15.4 (11.4, 19.4)	18.7 (14.9, 22.2)
High grade fever within 7 days of delivery	17.2 (13.1, 21.2)	11.9 (8.5, 15.1)	14.4 (11.1, 17.3)
Any complications during pregnancy [4]	9.1 (6.8, 11.6)	2.6 (1.6, 3.7)	5.6 (4.4, 6.7)
At least 1 antenatal care visit with skilled provider [5]	78.5 (73.8, 82.8)	77.3 (72.2, 81.4)	77.8 (73.4, 81.3)
Poor nutritional status [6]	10.7 (8.7, 13.1)	7.6 (5.1, 10.1)	9.1 (7.4, 10.6)
Received full antenatal package [7]	41.3 (34.4, 48.5)	47.3 (40.8, 54.9)	44.6 (38.9, 50.9)
Labor/delivery			
Delivery at health facility [8]	61.5 (50.6, 71.3)	59.1 (50.2, 67.4)	60.2 (50.9, 67.6)
Skilled birth attendant [5]	63.1 (53.4, 71.4)	60.9 (52.8, 68.5)	61.9 (54.0, 68.5)
Clean delivery kit	68.8 (57.1, 78.2)	64.5 (55.8, 72.0)	66.5 (57.4, 73.7)
Duration of ruptured membrane > 12 hours	27.9 (23.0, 32.8)	21.2 (17.2, 25.4)	24.3 (20.7, 27.6)
Foul smelling discharge during labor/delivery	24.1 (19.1, 28.7)	16.8 (12.7, 20.3)	20.2 (16.1, 23.6)
Any other complication during labor/delivery [9]	25.9 (22.5, 29.6)	18.3 (15.1, 21.6)	21.8 (19.2, 24.6)
Mother died	0 (0.0)	0.03 (0.004, 0.08)	0.02 (0.0, 0.04)
Characteristic	**Early-onset[1] % (LCL,UCL)[2] N=2100**	**Late-onset %(LCL,UCL) N=3153**	**Overall % (LCL,UCL) N=5253**
Infant			
Male	55.8 (51.0, 60.6)	58.4 (54.7, 62.5)	57.2 (54.3, 60.0)
Preterm [10]	23.5 (19.2, 27.9)	20.5 (17.2, 24.0)	22.0 (19.2, 24.8)
Low birthweight[11]	53.5 (49.4, 57.8)	46.8 (41.7, 52.1)	50.0 (46.1, 53.7)
Proper tying and cutting of umbilical cord [12]	53.5 (47.0, 59.4)	61.1 (54.5, 66.6)	57.6 (52.0, 62.4)
Proper application of antibiotic/antiseptic to umbilical cord [13]	44.7 (36.3, 53.0)	46.2 (38.8, 54.0)	45.5 (38.4, 52.1)
Delayed breathing or crying after birth [14]	20.3 (15.0, 25.1)	14.4 (11.1, 17.8)	17.2 (13.7, 20.2)
Ever initiated breastfeeding	77.0 (70.2, 82.5)	91.4 (89.3, 93.6)	84.7 (81.2, 87.9)
Given colostrum	43.9 (34.1, 55.3)	60.3 (51.6, 69.7)	52.8 (44.4, 61.9)
Any supplementation in first 3 days of life	29.4 (22.9, 36.4)	35.7 (28.0, 43.2)	32.8 (26.3, 38.9)
Any complications or illness since birth	50.2 (46.7, 54.0)	20.7 (17.3, 24.3)	34.4 (30.9, 37.9)
Integrated Management of Childhood Infection Signs			
Respiratory rate >=60 breaths per minute	47.6 (43.9, 51.2)	31.8 (27.2, 36.1)	39.1 (35.7, 42.2)
Severe chest in-drawing	10.5 (7.1, 14.0)	28.9 (23.0, 34.8)	20.4 (16.7, 24.4)
Axillary temperature >38.0`C (>100.4`F)	35.0 (27.3, 42.1)	33.6 (29.2, 38.2)	34.1 (29.1, 38.6)
Axillary temperature <35.5`C (<95.9`F)	21.8 (17.3, 26.3)	13.3 (8.7, 17.7)	17.3 (13.4, 20.8)

Characteristic	Early-onset[2] % (LCL,UCL)[3] N=2100	Late-onset %(LCL,UCL) N=3153	Overall % (LCL,UCL) N=5253
Movement only when stimulated or no movement	21.3 (16.6, 26.5)	16.3 (12.3, 21.3)	18.6 (14.7, 22.8)
Convulsions (confirmed by observation)	6.3 (4.2, 8.7)	2.7 (1.5, 4.1)	4.4 (3.1,5.8)
Poor feeding (confirmed by observation)	55.6 (49.5, 62.2)	26.2 (20.6, 33.0)	39.8 (33.9, 46.0)
Other clinical signs			
Apnea	7.0 (3.7, 10.7)	2.2 (1.5, 3.5)	4.4 (2.8, 6.4)
Cyanosis	13.1 (9.0, 17.9)	3.7 (2.1, 5.8)	8.1 (5.8, 10.8)
Bulging fontanelle	0.8 (0.2, 1.7)	0.5 (0.02, 0.7)	0.6 (0.3, 1.0)
Unable to cry	17.5 (11.8, 24.2)	8.4 (6.0, 11.1)	12.6 (9.3, 15.6)
Prolonged capillary refill	1.5 (0.2, 3.0)	0.2 (0.03, 0.5)	0.8 (0.1, 1.5)
Skin pustules	1.9 (1.0, 2.8)	8.0 (6.1, 10.2)	5.1 (4.1, 6.4)
Umbilical pus or redness	9.2 (6.6, 11.8)	6.7 (5.1, 8.7)	7.9 (6.3, 9.4)
Child Hospitalized	30.2 (23.0, 39.7)	24.6 (18.4, 31.8)	27.2 (21.2, 34.5)
Child Died	26.2 (14.6, 37.9)	19.6 (14.2, 26.2)	22.7 (15.5, 30.3)

[1] *Ureaplasma* attributable cases is defined as an individual infant who met the ANISA case definition, with a probabilistic assignment of *Ureaplasma* as the causative agent of illness, based on the estimated pLCM model results.

[2] Early onset is defined as illness in infants <3 days; late-onset is defined as illness in infants =>3 days of age and less than or equal to 59 days of age

[3] Lower credible limit, Upper credible limit

[4] Any complications during pregnancy is defined as having one of the following complications: high grade fever, foul smelling discharge, swelling of face or feet, excessive bleeding, or convulsions.

[5] A skilled provider or skilled birth attendant is defined as a qualified doctor, nurse, midwife, or paramedic.

[6] Poor nutritional status defined as mid upper arm circumference of less than 21.5 centimeters.

[7] Received full antenatal package defined as: 1. receipt of at least 2 antenatal visits from a community health worker; AND 2. Receipt of 2 tetanus injections during current pregnancy, OR 1 tetanus injection and at least 4 shots pre-pregnancy, OR at least 5 tetanus injections pre-pregnancy; AND 3. Receipt of at least one iron tablet or dose or iron syrup during current pregnancy.

[8] Delivery at a health facility is defined as being born at a public or private hospital, a maternity center, or clinic.

[9] Any other complication during labor/delivery is defined as excessive bleeding, convulsions, retained placenta, abnormal presentation, prolonged labor, or premature water breaking.

[10] Preterm is defined as an infant born <37 weeks gestational age; Term is defined as an infant born at => 37 weeks or greater.

[11] Low birth weight is defined as an infant weighing <2500 g at birth.

[12] Proper tying and cutting of umbilical cord is defined as use of an acceptable device for both cutting and tying the umbilical cord. Acceptable devices for cutting cords in the home must be boiled and be either thread, clip kit, knife, blade, or tongs. Acceptable devices for cutting cords in a hospital or health facility should be nurse/doctor's scissor or clip kit. Acceptable devices for tying cord in the home must be boiled and be either a clip, clip kit, thin rope brought by doctor, blade, or rubber band. Acceptable devices for tying a cord in a hospital or health facility is a clip or clip kit.

[13] Proper application of antibiotic or antiseptic to the umbilical cord differs by setting. In the home, antibiotic or antiseptic must be applied to the stump. In the hospital, antibiotic or antiseptic can be applied, or nothing can be applied.

[14] Delayed breath or crying is defined as a delay of either breathing or crying greater than 1 minute after delivery.

Table 8. Demographic and clinical characteristics of young *Ureaplasma* attributable cases[1], stratified by preterm status, Aetiology of neonatal Infections in South Asia (ANISA) Study, 2011-14

Characteristic	Preterm[2] % (LCL,UCL)[3] N=1183	Term %(LCL,UCL) N=3782	Overall % (LCL,UCL) N=4965[10]
Characteristic			
Maternal			
First live birth	30.8 (22.9, 38.6)	32.4 (27.7, 37.2)	29.5 (25.0, 34.4)
Completed secondary school	42.7 (33.1, 52.3)	41.0 (32.9, 50.2)	38.3 (30.2, 47.2)
Maternal age <20 years at delivery	5.1 (2.3, 8.2)	3.9 (2.4, 5.7)	3.8 (2.3, 5.7)
Pregnancy			
High grade fever during pregnancy	23.3 (16.3, 30.2)	17.1 (13.5, 20.6)	18.7 (14.9, 22.2)
High grade fever within 7 days of delivery	15.8 (10.8, 21.2)	12.8 (9.9, 15.5)	14.4 (11.1, 17.3)
Any complications during pregnancy [4]	6.1 (3.9, 8.5)	5.8 (4.4, 7.2)	5.6 (4.4, 6.7)
At least 1 antenatal care visit with skilled provider [5]	75.9 (67.9, 82.7)	78.1 (74.2, 81.6)	77.8 (73.4, 81.3)
Poor nutritional status [6]	11.9 (8.1, 16.6)	9.4 (7.6, 11.2)	9.1 (7.4, 10.6)
Received full antenatal package [7]	45.8 (36.7, 54.8)	48.0 (42.3, 54.2)	44.6 (38.9, 50.9)
Labor/delivery			
Delivery at health facility [8]	59.7 (47.5, 71.1)	60.7 (51.3, 68.7)	60.2 (50.9, 67.6)
Skilled birth attendant [5]	61.6 (50.1, 72.0)	62.2 (54.0, 69.2)	61.9 (54.0, 68.5)
Clean delivery kit	64.6 (50.6, 75.9)	66.7 (57.9, 73.4)	66.5 (57.4, 73.7)
Duration of ruptured membrane > 12 hours	22.9 (17.3, 29.2)	24.8 (21.0, 28.6)	24.3 (20.7, 27.6)
Foul smelling discharge during labor/delivery	19.7 (14.8, 24.5)	19.8 (15.8, 23.5)	20.2 (16.1, 23.6)
Any other complication during labor/delivery [9]	17.5 (13.3, 21.9)	22.6 (19.7, 26.0)	21.8 (19.2, 24.6)
Mother died10	---	---	0.02 (0.0, 0.04)
Characteristic	**Preterm[1] % (LCL,UCL)[2] N=1183**	**Term %(LCL,UCL) N=3782**	**Overall % (LCL,UCL) N=4965[10]**
Infant			
Male	52.2 (44.6, 59.1)	57.0 (54.0, 60.0)	57.2 (54.3, 60.0)
Early onset illness [11]	49.7 (38.9, 59.2)	44.4 (37.5, 50.8)	22.0 (19.2, 24.8)
Low birthweight[12]	70.2 (64.1, 76.4)	42.3 (38.7, 46.4)	50.0 (46.1, 53.7)
Proper tying and cutting of umbilical cord [13]	58.6 (50.7, 66.0)	57.0 (50.8, 62.3)	57.6 (52.0, 62.4)
Proper application of antibiotic/antiseptic to umbilical cord [14]	42.0 (31.4, 52.8)	46.3 (39.1, 53.4)	45.5 (38.4, 52.1)
Delayed breathing or crying after birth[15]	20.2 (14.8, 26.1)	15. 0 (11.6, 18.2)	17.2 (13.7, 20.2)
Ever initiated breastfeeding	79.1 (70.5, 86.4)	85.9 (83.0, 88.7)	84.7 (81.2, 87.9)
Given colostrum	51.5 (40.5, 63.4)	55.5 (47.2, 64.3)	52.8 (44.4, 61.9)
Any supplementation in first 3 days of life	31.6 (23.3, 40.8)	31.5 (25.0, 38.0)	32.8 (26.3, 38.9)
Any complications or illness since birth	32.6 (25.5, 40.3)	33.2 (29.0, 37.3)	34.4 (30.9, 37.9)
Integrated Management of Childhood Infection Signs			
Respiratory rate >=60 breaths per minute	36.4 (31.0, 41.8)	39.4 (35.2, 43.5)	39.1 (35.7, 42.2)
Severe chest in-drawing	14.9 (8.9, 21.8))	23.2 (18.8, 27.8)	20.4 (16.7, 24.4)
Axillary temperature >38.0`C (>100.4`F)	32.0 (24.3, 39.5)	36.2 (30.9, 40.9)	34.1 (29.1, 38.6)

Characteristic	Preterm[2] % (LCL,UCL)[3] N=1183	Term %(LCL,UCL) N=3782	Overall % (LCL,UCL) N=4965[10]
Axillary temperature <35.5`C (<95.9`F)	20.7 (13.6, 27.3)	13.7 (10.5, 16.8)	17.3 (13.4, 20.8)
Movement only when stimulated or no movement	24.4 (16.8, 32.6)	16.5 (12.9, 20.4)	18.6 (14.7, 22.8)
Convulsions (confirmed by observation)	2.4 (0.9, 4.4)	5.5 (3.8, 7.2)	4.4 (3.1,5.8)
Poor feeding (confirmed by observation)	53.1 (44.5, 61.9)	36.8 (30.8, 43.0)	39.8 (33.9, 46.0)
Other clinical signs			
Apnea	4.3 (1.9, 8.3)	5.0 (3.0, 7.0)	4.4 (2.8, 6.4)
Cyanosis	10.4 (5.1, 15.7)	7.3 (5.1, 9.7)	8.1 (5.8, 10.8)
Bulging fontanelle	0.6 (0.2, 1.1)	0.7 (0.3, 1.3)	0.6 (0.3, 1.0)
Unable to cry	16.7 (10.8, 24.9)	11.1 (7.8, 14.7)	12.6 (9.3, 15.6)
Prolonged capillary refill	0.7 (0.05, 2.4)	0.9 (0.2, 1.7)	0.8 (0.1, 1.5)
Skin pustules	5.8 (3.8, 8.5)	4.5 (3.3, 5.9)	5.1 (4.1, 6.4)
Umbilical pus or redness	7.1 (4.0, 10.7)	8.0 (6.5, 9.6)	7.9 (6.3, 9.4)
Child Hospitalized	28.8 (19.7, 39.8)	28.1 (22.1, 35.4)	27.2 (21.2, 34.5)
Child Died	28.6 (16.7, 41.8)	20.7 (14.2, 27.3)	22.7 (15.5, 30.3)

[1] *Ureaplasma* attributable cases is defined as an individual infant who met the ANISA case definition, with a probabilistic assignment of *Ureaplasma* as the causative agent of illness, based on the estimated pLCM model results.

[2] Preterm is defined as an infant born <37 weeks gestational age; Term is defined as an infant born at => 37 weeks or greater

[3] Lower credible limit, Upper credible limit

[4] Any complications during pregnancy is defined as having one of the following complications: high grade fever, foul smelling discharge, swelling of face or feet, excessive bleeding, or convulsions.

[5] A skilled provider or skilled birth attendant is defined as a qualified doctor, nurse, midwife, or paramedic.

[6] Poor nutritional status defined as mid upper arm circumference of less than 21.5 centimeters.

[7] Received full antenatal package defined as: 1. receipt of at least 2 antenatal visits from a community health worker; AND 2. Receipt of 2 tetanus injections during current pregnancy, OR 1 tetanus injection and at least 4 shots pre-pregnancy, OR at least 5 tetanus injections pre-pregnancy; AND 3. Receipt of at least one iron tablet or dose or iron syrup during current pregnancy.

[8] Delivery at a health facility is defined as being born at a public or private hospital, a maternity center, or clinic.

[9] Any other complication during labor/delivery is defined as excessive bleeding, convulsions, retained placenta, abnormal presentation, prolonged labor, or premature water breaking.

[10] N=288 infants had missing gestational age; 2 of the 4 mothers who died had infants with missing gestational age.

[11] Early onset is defined as illness in infants <3 days; late-onset is defined as illness in infants =>3 days of age and less than or equal to 59 days of age

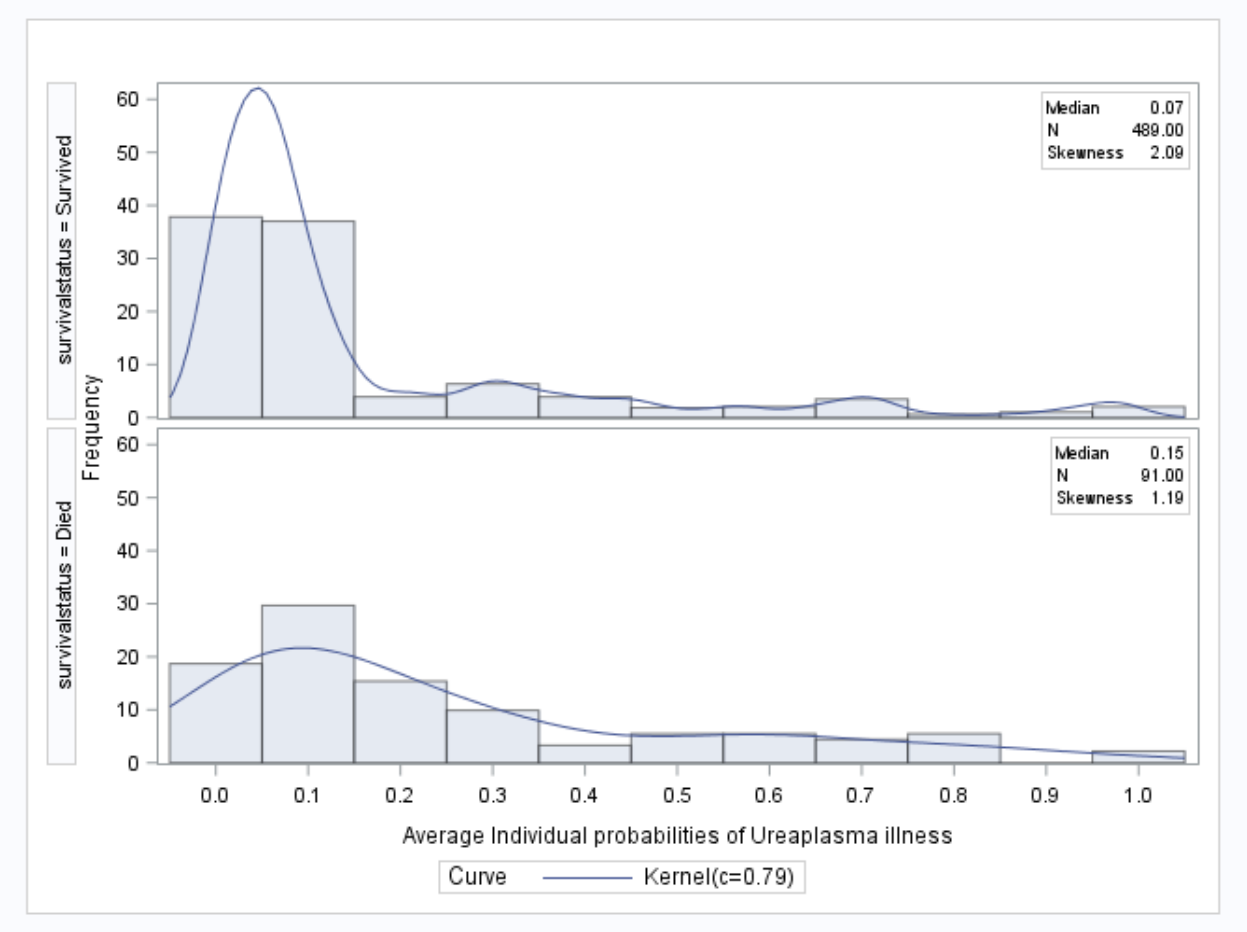
[12] Low birth weight is defined as an infant weighing <2500 g at birth.

[13] Proper tying and cutting of umbilical cord is defined as use of an acceptable device for both cutting and tying the umbilical cord. Acceptable devices for cutting cords in the home must be boiled and be either thread, clip kit, knife, blade, or tongs. Acceptable devices for cutting cords in a hospital or health facility should be nurse/doctor's scissor or clip kit. Acceptable devices for tying cord in the home must be boiled and be either a clip, clip kit, thin rope brought by doctor, blade, or rubber band. Acceptable devices for tying a cord in a hospital or health facility is a clip or clip kit.

[14] Proper application of antibiotic or antiseptic to the umbilical cord differs by setting. In the home, antibiotic or antiseptic must be applied to the stump. In the hospital, antibiotic or antiseptic can be applied, or nothing can be applied.

[15] Delayed breath or crying is defined as a delay of either breathing or crying greater than 1 minute after delivery.

Figure 8. Distribution of average individual probabilities1 of *Ureaplasma* infection among all cases[2] meeting the ANISA case definition, stratified by survived and died, Aetiology of Neonatal Infections in South Asia (ANISA) Study, 2011-14



[1] An average individual probability is the likelihood from the ANISA pLCM model that a given organism is the causative agent of illness for a given case.

[2]An ANISA case is defined as infant between 0 and 59 days of age that presented with one of the following signs, as confirmed by a physician: respiratory rate >=60/minute (fast breathing), severe chest in-drawing, axillary temperature either =>38°C (fever) or <35.5°C (hypothermia), movement only when stimulated or no movement at all, convulsions, or not feeding well confirmed on observation by study personnel.

CHAPTER 6: DISCUSSION

Defining etiology of illness is a challenging field of study, and even more so in neonates as their symptoms are non-specific. Given the paucity of data on etiology of neonatal infection, especially in low resource settings where neonatal mortality is high, the ANISA study sought describe the etiologic agents of neonatal infection. The ANISA study was conducted in countries with relatively high burden of neonatal disease and mortality, and high rates of refusal of referral for treatment/hospitalization, thereby providing the appropriate population to describe vital data on the etiologic spectrum of disease in young infants.

A large proportion of illness did not have an infectious etiology identified. Given this level of potential misclassification, our secondary objective was to estimate the prevalance of pathogens in three different subsets of infants meeting the WHO case definitions of critically ill, clinical severe infection, and isolated fast breathing in infants ages 3-59 days, in order to quantify if the more severe classifications were better at capturing infants with bacterial infection. We calculated the prevalence for every sign in the WHO case definition among infants meeting the clinical severe case definition to understand if any one danger sign was better at predicting infection. The data suggest it would be difficult to develop a clinical algorithm to distinguish bacterial from viral infections. Furthermore, we found none of the case definitions evaluated here distinguish well between identifiable infectious and non-identifiable causes of illness. While infants are likely being unnecessarily treated with antibiotics in these settings, given they meet the case definition but may have a viral etiology or no infectious etiology at all, there are infectious etiologies found even among the least severely ill infants. These data suggest

that there may be a substantial proportion of illness for which other interventions would be more effective, or there may be a number of unidentified pathogens causing illness.

While the proportion of infants with a bacterial etiology was highest in the most severe category, and lowest in the least severe category, there was still a non-zero proportion of those with bacterial etiology in every category. If the trials conducted in Africa and South Asia that evaluated the equivalency of simpler antibiotic regimens to the conventional regimens [46,47,49] had limited their study population to those with a bacterial etiology, simplified regimens may not have performed as well. However, in the absence of a method available to identify etiology of illness more readily in low resource settings, this analysis supports the current set of case definitions and the 2015 WHO guideline as appropriate tools for referral and treatment among those refusing referral.

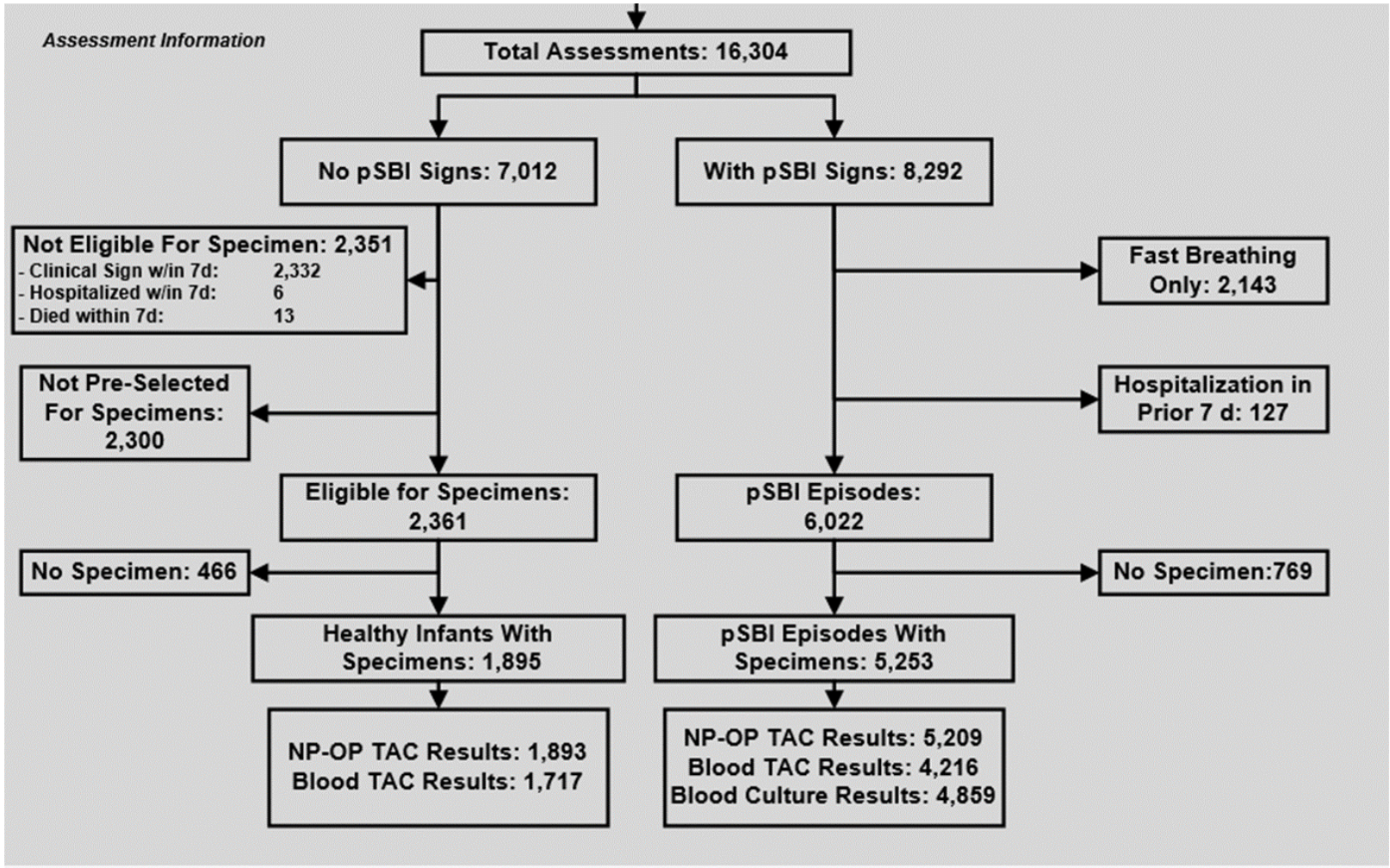
One of the most notable findings of the primary ANISA etiology study was *Ureaplasma* as the second most prevalent pathogen identified in this neonatal population. *Ureaplasma* has not been identified previously as a relevant pathogen for this age group, and the currently recommended regimens (typically gentamicin or amoxicillin) would not be effective against this agent, as *Ureaplasma* infection typically include macrolides or tetracyclines. [59] The description of the infant population with *Ureaplasma* attributed illness highlighted how different this population is from infants with *Ureaplasma* illness described previously in the literature. Given the severity of illness among infants in the *Ureaplasma* attributable population, either due the fact that *Ureaplasma* causes severe disease, or infants with *Ureaplasma* attributable illness had more severe outcomes due to treatment with antibiotics that are not effective against this agent, the fact that *Ureaplasma* is present in term infants, coupled with the complex media requirements that precluded growth on culture, and the fact that we did not identify another

viable explanation for the *Ureaplasma* finding, it is plausible to suggest that *Ureaplasma* has been under-detected in previous etiology studies.

Additional research into *Ureaplasma's* role in etiology of neonatal illness is needed, while being mindful of the unique requirements for diagnostics to accurately detect presence of this bacteria. Further research into *Ureaplasma* as a causative agent of illness in neonates, as well as the relationship between the presence of *Ureaplasma* with preterm and low birthweight status, would inform whether changes to current antibiotic regimens recommended by WHO for treatment of ill neonates are needed.

Lastly, improved diagnostics are key to further understanding causes of illness in neonates. Better point of care diagnostics for a range of pathogens need to be developed, and more awareness of *Ureaplasma* as a potential etiologic agent of illness in term and preterm neonates among clinicians is needed to ensure current appropriate diagnostics are utilized. From the methodologic perspective, better diagnostics for Bayesian pLCM methods should be developed to ensure precise and unbiased estimates are generated using these methods in a range of public health scenarios.

APPENDIX 1. FLOW DIAGRAM, AETIOLOGY OF NEONATAL INFECTIONS IN SOUTH ASIA (ANISA) STUDY, 2011-14



APPENDIX 2. OVERALL MOLECULAR POLYMERASE CHAIN REACTION DETECTIONS BY TAQMAN ARRAY CARDS (TAC) OF SEPSIS PATHOGENS AMONG INFANTS WITH POSSIBLE SERIOUS INFECTION BY CASE DEFINITION, AETIOLOGY OF NEWBORN INFECTIONS IN SOUTH ASIA (ANISA) STUDY, 2011-14

	Critically Ill[2]						**Clinical severe infection[3]**					
	NP/OP[1] TAC			**Blood TAC**			**NP/OP TAC**			**Blood TAC**		
	Positive ill infants N (%)	**Positive healthy infants N (%)**	**Odds Ratio**	**Positive ill infants N (%)**	**Positive healthy infants N (%)**	**Odds Ratio**	**Positive ill infants N (%)**	**Positive healthy infants N (%)**	**Odds Ratio**	**Positive ill infants N (%)**	**Positive healthy infants N (%)**	**Odds Ratio**
Number tested	**N = 977**	**N = 1893**		**N = 747**	**N = 1717**		**N = 3971**	**N = 1893**		**N = 3255**	**N = 1717**	
Pathogen												
Adenovirus	14 (1.4)	34(1.8)	0.8				58 (1.5)	34(1.8)	0.8			
Bordetella sp.	25 (2.6)	73(3.9)	0.7				203 (5.1)	73(3.9)	1.3			
Chlamydia pneumoniae	1 (0.1)	3(0.2)	0.5				9 (0.2)	3(0.2)	1.0			
Chlamydia trachomatis	2 (0.2)	5(0.3)	0.7				10 (0.3)	5(0.3)	1.0			
Cytomegalovirus	87 (9.4)	153(8.5)	1.1				245(6.8)	153(8.5)	0.8			
Escherichia coli	336(34.4)	461(24.4)	1.4	19 (2.5)	32(1.9)	0.5	958 (24.1)	461(24.4)	1.0	52 (1.6)	32(1.9)	0.8
Influenza A	12 (1.2)	13(0.7)	1.7				39 (1.0)	13(0.7)	1.4			
Influenza B	2 (0.2)	3(0.2)	1.0				29 (0.7)	3(0.2)	3.5			
group A *Streptococcus*				2 (0.3)	0(0)	/				12 (0.4)	0(0)	/
group B *Streptococcus*	92 (9.4)	123(6.5)	1.4	7 (0.9)	6(0.4)	2.3	309 (7.8)	123(6.5)	1.2	11 (0.3)	6(0.4)	0.8
Haemophilus influenzae				10 (1.3)	6(0.4)	3.3				25 (0.8)	6(0.4)	2.0
Human metapneumovirus	1 (0.1)	6(0.3)	0.3				20 (0.5)	6(0.3)	1.7			
Human parechovirus	2 (0.2)	12(0.6)	0.3				13 (0.3)	12(0.6)	0.5			
Klebsiella pneumoniae	274(28.1)	422(22.3)	1.3	24 (3.2)	33(1.9)	1.7	817 (20.1)	422(22.3)	0.9	51 (1.6)	33(1.9)	0.8
Mycoplasma pneumoniae	1 (0.1)	1(0.1)	1.0				9 (0.2)	1(0.1)	2.0			
Neisseria meningitidis				3 (0.4)	4(0.2)	2.0				4 (0.1)	4(0.2)	0.5
Parainfluenza virus type 1	2 (0.2)	7(0.4)	0.5				23 (0.6)	7(0.4)	1.5			
Parainfluenza virus type 2	0	7(0.4)	/				7 (0.2)	7(0.4)	0.5			

	Critically Ill[2]						Clinical severe infection[3]					
	NP/OP[1] TAC			Blood TAC			NP/OP TAC			Blood TAC		
	Positive ill infants N (%)	Positive healthy infants N (%)	Odds Ratio	Positive ill infants N (%)	Positive healthy infants N (%)	Odds Ratio	Positive ill infants N (%)	Positive healthy infants N (%)	Odds Ratio	Positive ill infants N (%)	Positive healthy infants N (%)	Odds Ratio
Number tested	**N = 977**	**N = 1893**		**N = 747**	**N = 1717**		**N = 3971**	**N = 1893**		**N = 3255**	**N = 1717**	
Pathogen												
Parainfluenza virus type 3	8 (0.8)	16(0.6)	1.3				51 (1.3)	16(0.6)	2.2			
Pseudomonas aeruginosa				6 (0.8)	7(0.4)	2.0				13 (0.4)	7(0.4)	1.0
Respiratory syncytial virus	62 (6.4)	25(1.3)	4.9				331 (8.3)	25(1.3)	6.4			
Rhinovirus / Enterovirus	223(22.8)	644(34.0)	0.7	24 (3.2)	49(2.9)	1.1	1185(29.8)	644(34.0)	0.9	105 (3.2)	49(2.9)	1.1
Rubella	5 (0.5)	5(0.3)	1.7				15 (0.4)	5(0.3)	1.3			
Salmonella sp.				14 (1.9)	30(1.8)	1.1				60 (1.8)	30(1.8)	1.0
Staphylococcus aureus				8 (1.1)	15(0.9)	1.2				21 (0.7)	15(0.9)	0.8
Streptococcus pneumoniae	286(29.3)	628(33.2)	0.9	16 (2.1)	20(1.2)	1.75	1436(36.2)	628(33.2)	1.1	44 (1.4)	20(1.2)	1.2
Ureaplasma sp.	152(15.6)	118(6.2)	2.5	3 (0.4)	6(0.4)	1.0	397(10.0)	118(6.2)	1.6	14 (0.4)	6(0.4)	1.0

	Bangladesh Isolated Fast Breathing [4]					
	NP/OP TAC			Blood TAC		
	Positive ill infants N (%)	Positive healthy infants N (%)	Odds Ratio	Positive ill infants N (%)	Positive healthy infants N (%)	Odds Ratio
Number tested	**N = 1161**	**N = 412**		**N = 847**	**N = 344**	
Pathogen						
Adenovirus	16 (1.4)	12(2.9)	0.5			
Bordetella sp.	12 (1.0)	6(1.5)	0.7			
Chlamydia pneumoniae	1 (0.9)	2(0.5)	1.8			
Chlamydia trachomatis	3 (0.3)	1(0.2)	1.5			
Cytomegalovirus	82 (8.1)	44(12.3)	0.7			
Escherichia coli	430(37.0)	123(29.9)	1.2	15 (1.8)	11(3.2)	0.6
Influenza A	10 (0.9)	3(0.7)	1.3			
Influenza B	6 (0.5)	0(0)	/			
group A *Streptococcus*				3 (0.4)	0(0)	/
group B *Streptococcus*	239 (20.6)	74(18.0)	1.1	4 (0.5)	6(1.7)	0.3
Haemophilus influenzae				7 (0.8)	3(0.9)	0.9

	Bangladesh Isolated Fast Breathing [4]					
	NP/OP TAC			Blood TAC		
	Positive ill infants N (%)	Positive healthy infants N (%)	Odds Ratio	Positive ill infants N (%)	Positive healthy infants N (%)	Odds Ratio
Number tested	**N = 1161**	**N = 412**		**N = 847**	**N = 344**	
Pathogen						
Human metapneumovirus	11 (1.0)	1(0.2)	5.0			
Human parechovirus	7 (0.6)	5(1.2)	0.5			
Klebsiella pneumoniae	308 (26.5)	101(24.5)	1.1	45 (5.3)	22(6.4)	0.8
Mycoplasma pneumoniae	2 (0.2)	0(0)	/			
Neisseria meningitidis				1 (0.1)	2(0.6)	0.2
Parainfluenza virus type 1	3 (0.3)	4(1.0)	0.3			
Parainfluenza virus type 2	2 (0.2)	2(0.5)	0.4			
Parainfluenza virus type 3	14 (1.2)	6(1.5)	0.8			
Pseudomonas aeruginosa				2 (0.2)	3(0.9)	0.2
Respiratory syncytial virus	43 (3.7)	3(0.7)	5.3			
Rhinovirus / Enterovirus	419(36.1)	190(46.1)	0.8	12 (1.4)	6(1.7)	0.8
Rubella	5 (0.4)	1(0.2)	2.0			
Salmonella sp.				21 (2.5)	8(2.3)	1.1
Staphylococcus aureus				6 (0.7)	10(2.9)	0.2
Streptococcus pneumoniae	593 (51.1)	248(60.2)	0.8	9 (1.1)	10(2.9)	0.4
Ureaplasma sp.	117 (10.1)	42(10.2)	1.0	2 (0.2)	2(0.6)	0.3

[1]NP/OP=Nasopharyngeal/oropharyngeal

[2]Critically Ill: NP/OP TAC: Number of ill infants tested for Cytomegalovirus N = 929; Number of healthy infants tested for Cytomegalovirus N = 1800; Blood TAC: Number of ill infants tested for *Neisseria meningitidis* N = 744

[3]Clinical severe infection: NP/OP TAC: Number of ill infants tested for Cytomegalovirus N = 3580; Number of healthy infants tested for Cytomegalovirus N = 1800; Number of ill infants tested for *Neisseria meningitidis* N = 3238

[4]Bangladesh Isolated Fast Breathing: NP/OP TAC: Number of ill infants tested for Cytomegalovirus N = 1013; Number of healthy infants tested for Cytomegalovirus N = 357; Number of ill infants tested for *Neisseria meningitidis* N = 844

APPENDIX 3. LIST OF PATHOGENS TESTED BY TAQMAN LOW DENSITY ARRAY (TAC) MOLECULAR PLATFORMS, BY TYPE OF SPECIMEN AVAILABLE FOR TEST, AETIOLOGY OF NEONATAL INFECTIONS IN SOUTH ASIA (ANISA) STUDY SITES, 2011-14

Pathogen	Type of specimen available for molecular test
E. coli/Shigella	both
Enterovirus	both
Group B *Streptococcus*	both
Klebsiella pneumoniae	both
Streptococcus pneumoniae	both
Ureaplasma spp.	both
Neisseria meningitidis	blood
Pseudomonas aeruginosa	blood
pan-*Salmonella*	blood
Staphylococcus aureus	blood
Group A *Streptococcus*	blood
pan-Haemophilus influenzae	blood
Human metapneumovirus	respiratory
Adenovirus	respiratory
Cytomegalovirus	respiratory
Influenza A	respiratory
Influenza B	respiratory
Parainfluenza virus 1	respiratory
Parainfluenza virus 2	respiratory
Parainfluenza virus 3	respiratory
Rubella	respiratory
Rhinovirus	respiratory
Mycoplasma pneumoniae	respiratory
Chlamydia trachomatis	respiratory
Chlamydia pneumoniae	respiratory
Bordetella pertussis I	respiratory
Respiratory syncytial virus	respiratory
Human parechovirus	respiratory

APPENDIX 4. PATHOGENS ISOLATED FROM BLOOD CULTURE AMONG INFANTS WITH POSSIBLE SERIOUS INFECTION IN BANGLADESH, BY AGE OF ONSET AND CASE DEFINITION, AETIOLOGY OF NEONATAL INFECTIONS IN SOUTH ASIA (ANISA) STUDY, 2011-14

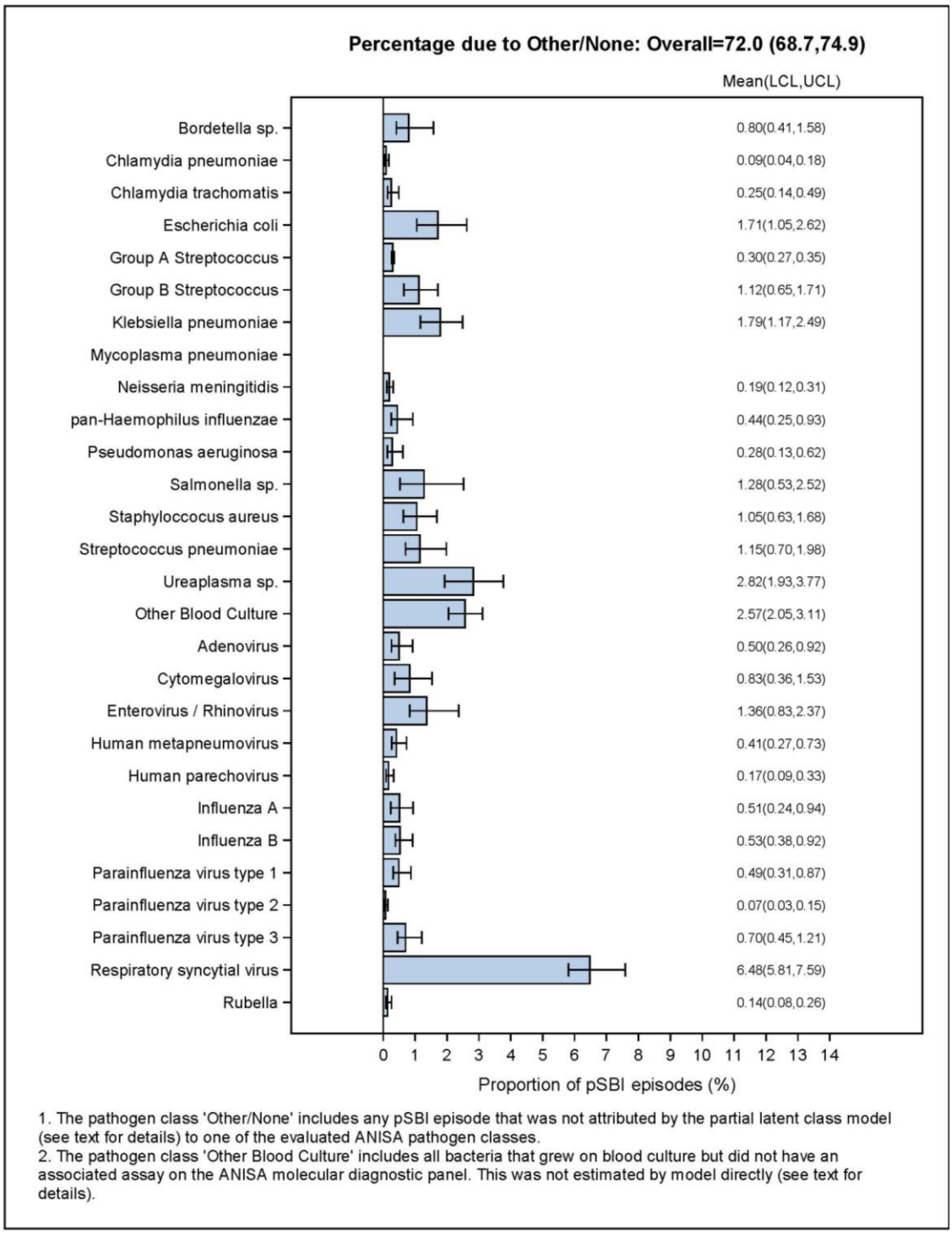
	Early Onset[1]		Late Onset[2]		
Pathogen	**Bangladesh Clinical Severe Infection (n=523)**	**Bangladesh Isolated Fast Breather (n=473)**	**Bangladesh Clinical Severe Infection (n=702)**	**Bangladesh Isolated Fast Breather (n=529)**	**Total Number**
Subtotal gram positive	9	3	6	0	**18**
Enterococcus faecium					
group A *Streptococcus*	1	3	3		**7**
group B *Streptococcus*	5				**5**
Staphylococcus aureus			1		**1**
Streptococcus oralis	1				**1**
Streptococcus pneumoniae	2		2		**4**
Subtotal gram negative	4	1	8	1	**14**
Acinetobacter					
Burkholderia cepacia					
Citrobacter koseri					
Edwardsiella tarda			1		**1**
Enterobacter sakazakii				1	**1**
Escherichia coli	2	1	2		**5**
Klebsiella pneumoniae	1				**1**
Morganella morganii			1		**1**
Neisseria meningitidis			4		**4**
Proteus mirabilis					
Pseudomonas pseudomallei	1				**1**
Salmonella enterica					
Serratia marcescens					
Total	**13**	**4**	**14**	**1**	**32**

[1] Early onset is defined as < 3 days of life
[2] Late onset is defined as => 3 days and <60 days of life

APPENDIX 5. PATHOGENS ISOLATED FROM BLOOD CULTURE AMONG INFANTS MEETING THE CRITICALLY ILL AND CRITICAL SEVERE INFECTION CASE DEFINITIONS, AETIOLOGY OF NEONATAL INFECTIONS IN SOUTH ASIA (ANISA) STUDY, 2011-14

Pathogen	Critically Ill Infant (N=992)	Clinical Severe Infection (N=4000)	Total
Subtotal gram positive	9	26	35
Enterococcus faecium	1		1
group A *Streptococcus*		11	11
group B *Streptococcus*	2	4	6
Staphylococcus aureus	4	8	12
Streptococcus oralis			0
Streptococcus pneumoniae	2	3	5
Subtotal gram negative	29	34	63
Acinetobacter	2	1	3
Burkholderia cepacia	1	1	2
Citrobacter koseri		1	1
Edwardsiella tarda	1		1
Enterobacter sakazakii	2	1	3
Escherichia coli	7	13	20
Klebsiella pneumoniae	10	6	16
Morganella morganii		1	1
Neisseria meningitidis	1	4	5
Plesiomonas shigelloides		1	1
Proteus mirabilis		1	1
Pseudomonas aeruginosa	1		1
Pseudomonas pseudomallei	2	1	3
Salmonella enterica		2	2
Serratia marcescens	2	1	3
Total	**38**	**60**	**98**

APPENDIX 6. ESTIMATES FROM A PARTIAL LATENT CLASS MODEL OF THE PREVALENCE OF PATHOGENS CAUSING POSSIBLE SERIOUS BACTERIAL INFECTION, AETIOLOGY OF NEONATAL INFECTIONS IN SOUTH ASIA (ANISA) STUDY, 2011-2014[68]



1. The pathogen class 'Other/None' includes any pSBI episode that was not attributed by the partial latent class model (see text for details) to one of the evaluated ANISA pathogen classes.
2. The pathogen class 'Other Blood Culture' includes all bacteria that grew on blood culture but did not have an associated assay on the ANISA molecular diagnostic panel. This was not estimated by model directly (see text for details).

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